Post transcriptional control of HIV-1 latency by the PKR and p53 pathways

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December 2016

"A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science"

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ABSTRACT

English

Latency represents the major obstacle currently faced in the development of a curative HIV therapy. Latency is responsible for the persistence of HIV in treated individuals and can be established in different cell types. It has been shown that components of intrinsic immunity play a critical role in the establishment and maintenance of latency.

Protein Kinase R (PKR) is an interferon stimulated gene (ISG). It is a sensor of pathogen associated molecular patterns (PAMP) and is activated by double stranded RNA. Activated PKR inhibits the production of viral proteins in HIV infected cells. PKR is transiently activated at the beginning of HIV infection but is rapidly deactivated during active replication. The tumour suppressor p53 is a transcription factor that can be activated by various cellular stresses such as DNA damage, ultra violet light, ionising radiations, ribonucleotide starvation or oxidative stress. In response to stress p53 can promote the transcription of antiproliferative and proapoptotic genes. Interplay between PKR and p53 pathway result in the inhibition of the viral replication of several viruses including HIV.

We hypothesised that PKR activation and inhibition of translation initiation is a contributing factor in the establishment of HIV latency in specific cell types through different pathways and can be targeted for the development of new therapies.

To address the reactivation pattern and the role of the PKR and p53 pathways during latency we used an HIV-1 latent model. This model is based on two cell lines: $CD4^+$ T lymphocytes (CEM-T4) and Monocyte Derived Macrophages or MDM (THP-1). The model consists of a modified HIV-1 provirus, integrated within the host cell DNA. Modifications include a GFP reporter fused to the Gag viral protein used to monitor levels of reactivation. To

induce reactivation, cells were treated with Latency Reversing Agents (LRAs) targeting transcription through the Nuclear Factor κB and Protein Kinase C pathways.

Our results show that some LRAs modify the expression and activation of PKR in certain cell types. These results suggest that the PKR pathway could play a role in the establishment of latency in specific cellular reservoirs and novel LRAs targeting PKR could be identified.

Français

La latence représente un des principaux obstacles au développement d'une thérapie curative contre le VIH. Elle est responsable de la persistance du VIH chez les individus suivant un traitement antirétroviral et se développe dans plusieurs types cellulaires. Certaines composantes du système immunitaire intrinsèque et innée ont un rôle critique dans l'établissement et le maintien de la latence.

La protéine Kinase R (PKR) est un gène stimulé par l'interféron (ISG). PKR est un senseur des motifs moléculaires associés pathogènes (PAMPS) et est activée par l'ARN double brin. Quand PKR est activée, elle inhibe la production des protéines virales dans les cellules infectées par le VIH. PKR est transitoirement activée au début de l'infection par le VIH puis est rapidement inhibée lors de la réplication active du virus. Le suppresseur de tumeur p53 est un facteur de transcription qui peut être activé par différents stimuli tels que les dommages à l'ADN, les Ultraviolets, les radiations ionisantes, la carence en ribonucléotides ou le stress oxydatif. En réponse au stress, p53 permet la transcription de gènes antiprolifératifs et proapoptotiques. La communication entre les voies de signalisations de PKR et de p53 inhibent la réplication de plusieurs virus dont le VIH.

Nous avons émis l'hypothèse que l'activation de PKR et l'inhibition de l'initiation de la traduction sont des facteurs contribuant à l'établissement de la latence du VIH dans certains types cellulaires par différentes voies de signalisation qui peuvent être ciblées pour le développement de nouvelles thérapies.

Pour élucider les profils de réactivation et le rôle de PKR et de p53 au cours de la latence, nous avons utilisé un modèle de latence du VIH-1. Ce modèle est basé sur deux lignées cellulaires de Lymphocytes T $CD4^+$ (CEM-T4) et de Macrophages Dérivés de Monocytes ou MDM (THP-1). Ce modèle consiste en un provirus génétiquement modifié, intégré au génome de la cellule hôte. Les modifications génétiques apportées incluent un gène rapporteur GFP fusionné à la protéine Gag, utilisé pour contrôlé les niveaux de réactivation du virus. Pour induire la réactivation, des agents d'inversion de la latence (LRA) ciblant les voies de signalisation NF- κB et PKC, ont été utilisés.

Nos résultats ont montré que les LRA modifient l'expression et l'activation de PKR dans certains types cellulaires. Ces résultats suggèrent que la voie de PKR jouerait un rôle essentiel dans l'établissement de la latence dans des types cellulaires spécifiques et que de nouveaux LRA ciblant PKR pourraient être identifiés.

ACKNOWLEGEMENTS

I would like express my gratitude to Doctor Anne Gatignol for giving me the opportunity to work in her laboratory on this exciting and new project. She was very supportive and always drove me to better my research and coursework. I learned more than I could have wished for in terms of techniques, writing and presenting my results that I now feel confident and prepared for what future challenges in my career. I thoroughly enjoyed the past two years spent in the laboratory and this experience was greatly improved by all my amazing co-workers. I would like to thank our lab manager Aïcha Daher for being available and the time she invested in training and advising me. I would also like to express my gratitude to Dr. Robert Scarborough for sharing his experience, advice and pipettes with me. Special thanks must also go to Dr. Elodie Rance for her help with the FACS experiments and the more than welcome help and company in the P3. I should also mention PhD candidate Sergio Alpuche whom could always be counted on for his support and friendship. I would like to thank all the other current and past members of the lab I had the pleasure and honour to meet and work with Roman Radetskyy, Kelsey Adams and Olivier Del Corpo. Finally, I would like to wish a smooth academic year and successful research to new members Craig McCullogh and Ryan Goguen.

PREFACE AND CONTRIBUTION OF AUTHORS:

This thesis was written under the supervision of Dr. Anne Gatignol. The introduction was written with the help of Dr. Gatignol and Aïcha Daher who provided many of the references. Figures 1 to 6 were re-designed by Roman Labbé from data and figures referenced under each figure.

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LIST OF ABBREVIATIONS

ADAR1: Adenosine Deaminase acting on RNA1

AIDS: Acquired Immuno Deficiency Syndrome

APOBEC3: Apolipoprotein B mRNA editing enzyme

CA: HIV capsid

cART: combined Antiretroviral Therapy

cDNA: complementary DNA

Cdc2: Cell division cycle 2 protein

CRS: the Cis-acting Repressive Sequences

dsRBD: Double stranded RNA binding domain

gp: glycoprotein

HAART: Highly active antiretroviral therapy

HCV: Hepatitis C virus

HIV: Human immunodeficiency virus

HSV: Herpes Simplex Virus

HTLV: Human T Lymphotropic Virus

IFN: Interferon

INT: HIV integrase

INS: Instability RNA sequences

IRES: Internal ribosomal entry site

IRF3: Interferon regulatory factor 3

JAK: Janus kinase

LRA: Latency Reversing Agent

LTR: Long Terminal Repeat

LT: Lymphocyte T

MA: HIV Matrix

MAPK: Mitogen activated protein kinase

MDM: Monocytes-derived Macrophages

MDA-5: Tumour Melanoma Differentiation Associated suppressor 5

NC: HIV Nucleocapsid

Nef: HIV Negative regulatory factor

NF-κB: Nuclear factor kappa-light chain enhancer of activated B cells

NNRTI: Non-nucleoside reverse transcriptase inhibitor

NRTI: Nucleoside reverse transcriptase inhibitor

PACT: PKR activator

PAMP: Pathogen associated molecular patterns

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate buffer saline

pDC: Plasmacytoid dendritic cells

PE: Psi elements SLIP: the TTTTTT slippery site

PIC: HIV preintegration complex

PKR: Protein Kinase RNA-activated

pre-miRNA: pre-microRNAs

PROT: HIV protease

RAX: PKR activator mouse homolog

Rev: HIV Regulator of viral expression

RHA: RNA helicase A

RIG-I: Retinoic acid-inducible gene

RNAi: RNA interference

RRE: HIV Rev-responsive element

RT: HIV reverse transcriptase

SAMHD1: SAM domain and HD domain-containing protein

siRNA: Small interfering RNA

SIV: Simian immunodeficiency virus

SIVcpz: chimpanzee Simian Immunodeficiency Virus

SIVgor: gorilla Simian Immunodeficiency Virus

SIVsmm: sooty mangabey Simian Immunodeficiency virus

Slfn: Schlafen

STAT: Signal transducer and activator of transcription

SUMO: Small Ubiquitin-like Modifiers

TAR: Trans-Activation Response element

Tat: Trans-activator of transcription

TBS-T: Tris buffer saline containing 1 % tween20

TRBP: TAR RNA Binding Protein

TRIM5: Tripartite motif-containing protein 5

Vif: HIV viral infectivity factor

Vpr : HIV viral protein R

Vpu : HIV-1 viral protein U

Vpx : HIV-2 viral protein X

VSV: Vesicular Stomatitis Virus

VV: Vaccinia Virus

WHO: World Health Organization

ZBD: Z-DNA binding domain

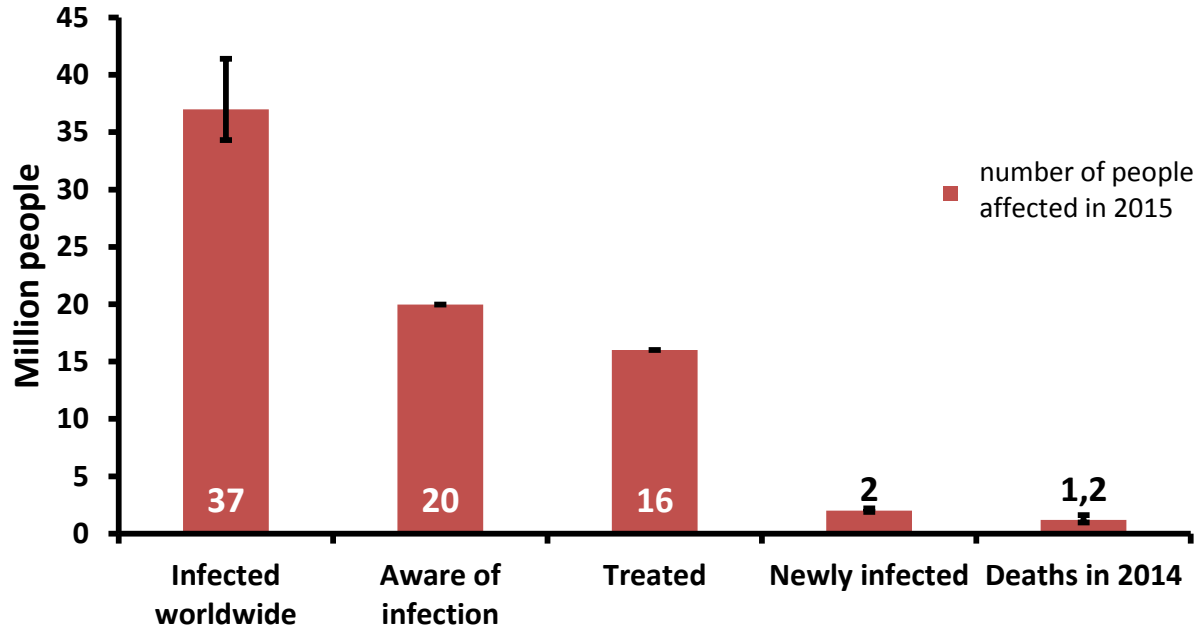
INTRODUCTION

1- The Human Immunodeficiency Virus HIV

The HIV Pandemic

The Human Immunodeficiency Virus or HIV is a family member of the *Retroviridae* and is responsible for the development of the Acquired Immunodeficiency Syndrome or AIDS. According to the WHO (WHO, 2015), the HIV pandemic affects 37 million people worldwide. Only 54% of seropositive people are aware of their infection and around 43% are currently treated. It is estimated that 2 million people are newly infected every year and 1.2 million HIV-related deaths have been recorded in 2014. The burden of HIV infected populations varies greatly between countries and the most affected region is sub-Saharan Africa with 70% of the cases worldwide. These figures are highlighted in **Figure 1.**

Figure 1. The HIV Pandemic



Data collected by the WHO on key features of the HIV Pandemic (2015).

Highly active antiretroviral therapy (HAART) has changed HIV infection from a deadly to a chronic disease. There is currently no cure to HIV infection. Better detection, an increase access to available therapies, especially in under-developed countries and the development of novel therapies are still required to control the HIV global pandemic.

HIV-1 and HIV-2

HIV is known to present a very high genetic variability and HIV viruses are divided in types and subtypes. The two main types of HIV are HIV type 1 (HIV-1) and HIV type 2 (HIV-2) (Nyamweya et al., 2013). HIV-1 is the type responsible for the world pandemic with approximately 95% of the cases worldwide. HIV-2 is usually endemic to West Africa with few cases reported outside of the region (WHO, 2015).

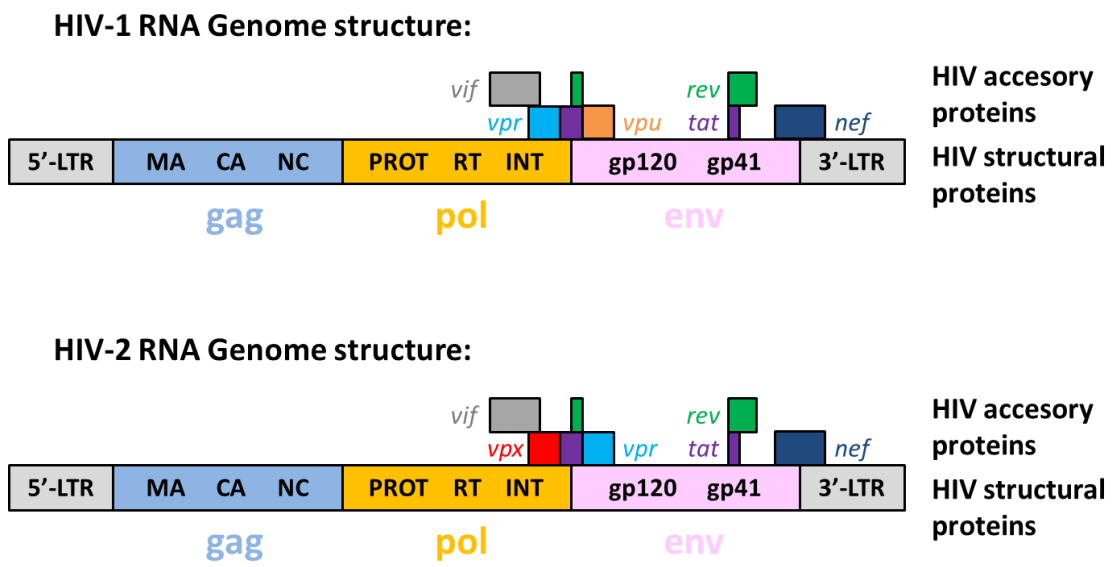
HIV-1 and HIV-2 are very similar in terms of genetic structure, intracellular replication and have similar outcomes in terms of clinical consequences. If untreated, both HIV type infections result in the development of AIDS. HIV-2 is less readily transmitted and is less likely to evolve towards AIDS with many patients remaining long term non progressors (Nyamweya et al., 2013).

HIV-1 and HIV-2 originate from different cross species transmissions. HIV-1 groups M, N and O originated from independent chimpanzee Simian Immunodeficiency Virus (SIVcpz) and HIV-1 group P originated from gorilla Simian Immunodeficiency Virus (SIVgor). HIV-2 finds its origins in sooty mangabey Simian Immunodeficiency virus (SIVsmm) (Sharp and Hahn, 2011).

The majority of cases of HIV worldwide are caused by HIV-1 group M, which stands for group Major. Group M is subdivided into nine subtypes denominated by the letter A, B, C, D, F, G, H, J, K (Hemelaar, 2012). This genetic diversity can impact disease progression, risk of transmission and response to treatment (Pant Pai et al., 2012).

Although HIV-1 and HIV-2 have very similar gene layout, some differences can be observed. The most notable difference is that HIV-1 genome codes for the accessory protein Vpu whereas HIV-2 codes for the protein Vpx. The genomic structure of HIV-1 and HIV-2 RNA genomes and the proteins they encode are presented in **Figure 2.**

Figure 2. Structure of the HIV-1 and HIV-2 RNA genomes



Modified from (Matheson et al., 2016)

The HIV-1 RNA genome contains 9 genes with three structural genes, gag, pol, and env encoding 8 structural proteins, and six genes coding for the accessory proteins Vif, Vpr, Vpu, Rev, Tat, and Nef. The HIV RNA genome also harbours distinctive RNA structures such as the Long Terminal Repeats (LTRs), the Trans-Activation Response element (TAR), the Rev Response Element (RRE), the PE Psi elements, the TTTTTT slippery site (SLIP), the Cis-acting Repressive Sequences (CRS) and the Inhibitory/Instability RNA sequences (INS) essential to the virion's replication cycle (Knoepfel and Berkhout, 2013).

A mature HIV virion is 120 nm in diameter and carries two copies of the RNA genome enclosed in the viral capsid formed by the protein p24. The RNA is stabilised by structural proteins such as the nucleocapsid (NC) and p7. The capsid also contains essential enzymes

needed for the maturation of the virus such as the protease (PROT), the reverse transcriptase (RT) required for reverse transcription and the integrase (INT) required for the integration of the viral genome (Campbell and Hope, 2015). The capsid is then enclosed in a spherical matrix formed by the protein p17 (Fiorentini et al., 2006). The matrix is finally coated in a lipid bilayer taken from the cell membrane during the virion's budding. The membrane accommodates the viral proteins glycoprotein (gp) 120 and gp41, which form hexameric envelope protein (gp160) composed of three gp41 anchoring the structure in the membrane attached to three gp120 (Yoon et al., 2010b, Bar and Alizon, 2004).

The HIV-1 tropism and replication cycle

Viral tropism refers to the type of cell that a virus can infect and use to replicate. HIV is known to infect cells such as T Lymphocytes $CD4^+$, Macrophages and Microglial cells. HIV tropism is defined by its protein gp120, which specifically binds the glycoprotein co-receptor CD4 and mediate entry through chemokine co-receptors (Clapham and McKnight, 2001).

The type of chemokine co-receptor used for entry is used to define the type of HIV-1 strain. The Macrophage-tropic or R5 uses the β-chemokine co-receptor CCR5 for entry. The Lymphocyte-tropic or X4 uses the α-chemokine co-receptor CXCR4 for entry. Both R5 and X4-tropic strains are found in seminal fluid but the R5 strain is the strain predominantly transmitted during sexual intercourse through unknown selective processes. The X4 strain usually appears during late stages of infection and aggressively replicates in T lymphocytes, causing the severe drop in lymphocytes observed during the development of AIDS. Dual-tropic strains or R5X4 exist but are considered as transitional strains (Blanpain et al., 2002, Berger et al., 1998). HIV has been shown to infect astrocytes although HIV replication seems to be restricted in this cell type and is non-productive *in vivo*. However infection of astrocytes contributes to HIV associated

dementia (HIVD) and affects up to 20% of infected adults (Gorry et al., 2003). Human Dendritic Cells (DCs) are resistant to productive HIV infection. However, DCs can internalise HIV virions and can transfer HIV to $CD4^+$ cells during membrane-membrane contacts (Cavrois et al., 2007, Jochems et al., 2015, Menager and Littman, 2016).

HIV replication cycle can be broken down into several steps, each involving a specific set of viral and cellular factors.

Entry: During this phase, the HIV mature virion attaches its target receptor via its envelope protein gp160. The gp120 trimer strongly interacts with the CD4 target receptor inducing a conformational change and exposing the chemokine co-receptor binding domain of gp120 and recruitment of the chemokine co-receptor. The virion is then strongly attached to the cell membrane and the N-terminus of the gp41 is inserted into the cellular trans-membrane domain. This induces a conformational change in the extracellular structure of gp41, bringing the viral and cellular membranes closer together. The membranes fuse and the viral core surrounded by the capsid and matrix is then released into the cytoplasm (Wilen et al., 2012, Chan and Kim, 1998, Wyatt and Sodroski, 1998).

Reverse transcription: After the entry inside the cell, the core is transported towards the nucleus via microtubule transport. Partial uncoating of the matrix takes place during this step although the timing of this process remains unclear due to the vulnerability of the viral matrix to experimental manipulations (Arhel, 2010). The RT then begins transcribing a complementary circular DNA (cDNA) strand inside the core. During the reverse transcription, the RNAse activity of the RT degrades the RNA strand while copying it. The RNA polymerase function of the RT then creates a complementary cDNA strand using the antisense cDNA as a template. This process is highly error prone and is responsible for the genetic diversity of HIV and subsequently for the rapid appearance of resistance mutations against treatments. Once the reverse transcription is

completed, the viral cDNA genome and viral and host proteins form a Pre-Integration complex (PIC). The integrase cleaves the 3' ends of the DNA and generates a 5' overhang. The PIC is then transported to the nucleus through a nuclear pore and binds to the host DNA (Hu and Hughes, 2012, Zheng et al., 2005).

Integration: The integrase binds as a dimer to the LTR region of the double stranded cDNA in the PIC. The nuclease activity of the HIV integrase through the hydroxyl group attack of the 3' end host DNA. The viral DNA then becomes integrated into the host DNA leaving a few base pair gaps between the 3' and 5'end. It is then speculated that the gaps left after DNA integration are then filled by the cellular DNA repair machinery but have yet to be investigated (Craigie and Bushman, 2012, Zheng et al., 2005).

Transcription and translation: During transcription, the viral DNA is transcribed into viral mRNA, which is then doubly spliced, exported out of the nucleus and translated into the accessory proteins Tat, Rev and Nef. Tat and Rev are then imported to the nucleus and while Tat stimulates the expression of the HIV provirus, the accumulation of the Rev protein allows it to bind to the HIV RRE RNA and promotes the nuclear export of singly spliced and unspliced RNA. The exported full length HIV will then serve as copies of the HIV RNA genome or as templates to produce the structural HIV proteins Gag and GagPol, whereas the singly spliced RNA will be translated to produce Env (Karn and Stoltzfus, 2012).

Assembly: The assembly of new viral particles occurs at the plasma membrane of the host cell. The Env polyprotein is processed through the endoplasmic reticulum, where the cell enzyme Furin cleaves it into the mature gp41 and gp120 proteins which are then transported to the plasma membrane. The Gag and Gag-Pol polyproteins also agglomerate at the inner plasma membrane. Finally the HIV genomic RNA is recruited to the plasma membrane where it starts budding with the other protein components into a new virion (Sundquist and Kräusslich, 2012).

Release and maturation: once the virion buds off from the plasma membrane, it is still immature. The polyprotein Gag is cleaved into individual Matrix, Capsid and Nucleocapsid proteins by the viral protease. The different proteins assemble into the Capsid, Matrix and nucleocapsid structures to form a new infectious HIV mature virion (Sundquist and Kräusslich, 2012).

Clinical features and therapies

Clinical Features

HIV best known clinical feature is the Acquired Immunodeficiency Syndrome (AIDS). However this condition only arises in the late stages of HIV infection.

According to the WHO (WHO, 2015) a person infected with HIV may experience flu-like symptoms 2-4 weeks after infection. At this stage HIV might not be detected by standard HIV testing, however the individual will be highly contagious and may spread the infection to others.

The immune system then clears the majority of the viral infection and the disease enters its chronic phase. During the chronic phase, the virus is still present but reproduced at low rate. If untreated, the seropositive individual will still be contagious and may spread the disease to others. This phase may last up to a decade although this period greatly varies from one individual to another.

If the individual is untreated during the chronic phase, most individuals will eventually progress to AIDS. AIDS is a severe loss of cellular immunity leading to the development of opportunistic infections and malignancies. The main marker used to diagnose AIDS is the $CD4^+$ T cell count. When a patient is diagnosed with an HIV infection and a $CD4^+$ cell count bellow 200 cells. μL^{-1} the diagnostic recommended by the CDC directives is AIDS. A list of AIDS

associated diseases was also published by the CDC in (1987) and revised in (1993) which can also be used for diagnosis. Some of the conditions listed include malignancies such as Kaposi's sarcoma or Burkitt's lymphoma, encephalopathy or wasting syndrome (cachexia) opportunistic infections such as cytomegalovirus disease or toxoplasmosis in the brain.

Available therapies

Even though there is no cure available against HIV, treatments have been developed and have changed HIV infection from a deadly disease into a chronic infection (Brechtl et al., 2001, Moore and Chaisson, 1999). HIV's genetic variability has been one of the main challenges in the development of treatments and there is no available HIV vaccine to date. To reduce the likelihood of the appearance of genetic mutations conferring resistance to the virus, drugs are used in combination. This practice is known as Highly Active Antiretroviral Therapy (HAART) or combined Antiretroviral Therapy (cART). Different classes of drugs are used, usually comprising a Reverse Transcriptase inhibitor, constituting the backbone of the therapy (WHO, 2016).

Entry inhibitors: Entry inhibitors or Fusion inhibitors inhibit the HIV virion binding to the target cells. This class of drugs usually functions by interacting with receptors involved in HIV binding. For instance, Maraviroc (Selzentry© or Celsentri©) inhibits binding of gp120 to CCR5 by binding to CCR5 and Enfuvirtide (Fuzeon©) binds to gp41 and inhibits its ability to modify its structure to bring the cell and viral membranes together (Kuritzkes, 2009).

Reverse transcriptase inhibitors:

Nucleoside RT inhibitors NRTIs: This class of drugs inhibits the RT enzyme by competing with the natural deoxynucleoside required by the virus to synthesise its viral DNA.

These nucleoside analogues cannot form a phosphodiester bond with the next nucleoside incorporated by the reverse transcriptase leading to the chain termination. This class of drugs was the first antiretroviral drug to be introduced as HIV treatment and include Sidovudine also known as AZT (Retrovir©) and Lamavudine also known as 3TC (Zeffix© and Epivir©) (De Clercq, 1998).

Nucleotide RT inhibitors NtRTIs: Nucleotide RT inhibitors work in the same manner as NRTIs and both are classified as competitive substrate inhibitors. NtRTIs include drugs such as Tenofovir (Viread©) and Adefor (Preveon©) (Akanbi et al., 2012).

Non-Nucleoside RT inhibitors NNRTIs: This class of inhibitors inhibits the Reverse Transcriptase by direct binding. They are non-competitive inhibitors and are not incorporated to the DNA. Instead they block the movements of the enzyme therefore inhibiting viral DNA synthesis. Efavirenz (Sustiva© or Stocrin©) and Rilpivirin (Edurant©) are both approved NNRTIs (Pauwels, 2004).

Protease inhibitors: This class of drugs inhibits the viral protease activity by direct binding. This results in the production of non-infectious viruses since the protease cannot cleave the polyprotein precursors essential for the maturation of the virus. This class of drug includes Saquanavir (Fortovase or Invirase©) and Amprenavir (Agenerase©) (Flexner, 1998).

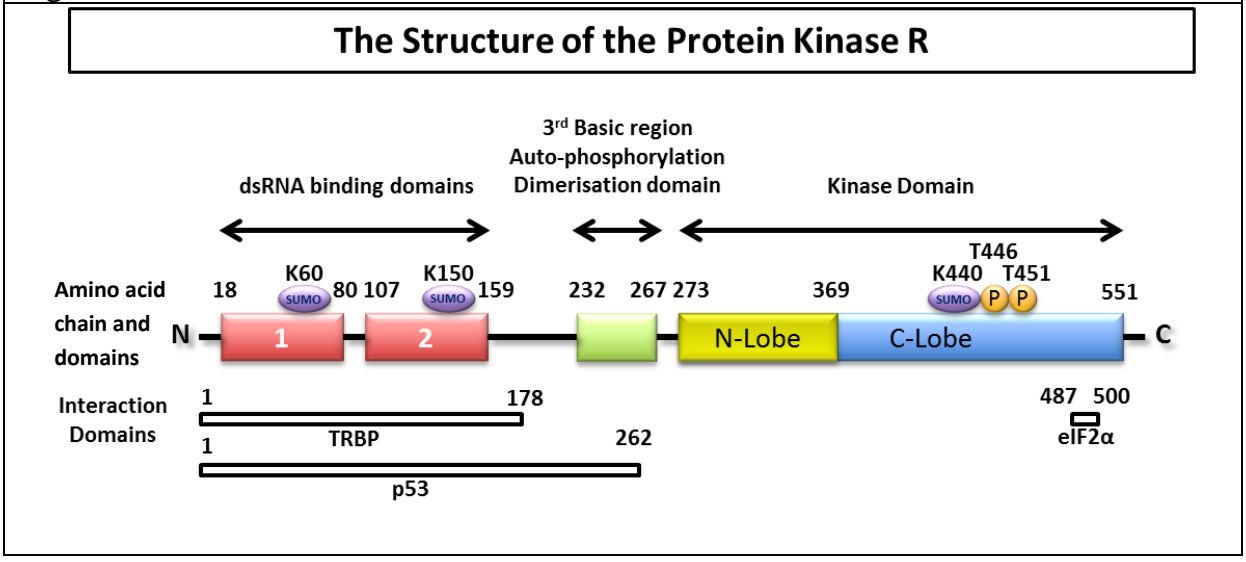
Integrase inhibitors: Integrase Strand Transfer Inhibitors (INSTI) block the activity of the HIV integrase preventing the virus from integrating its DNA into the host DNA. INSTIs includes drugs such as Dolutegravir (Tivicay©), Elvitegravir (Vitekta©) and Raltegravir (Isentress©) (Adams et al., 2012).

2- The Protein Kinase R and the PKR pathway

The Interferon-induced Double stranded RNA-activated Protein Kinase

The IFN-induced double stranded RNA-activated protein kinase or Protein Kinase R (PKR) is a serine/threonine protein kinase of the translation Initiation factor 2α (eIF-2α). The human PKR gene was isolated from cDNA libraries expressing a protein strongly induced by IFN (Clemens et al., 1993, Meurs et al., 1990, Hovanessian, 2007). Its gene, EIF2AK2 codes for a 2.5 kilobase messenger RNA which produces a 551 amino acid protein (Kuhen et al., 1996, Meurs et al., 1990, Feng et al., 1992). The PKR protein structure comprises several remarkable landmarks. PKR possesses two 65 amino acid long double stranded RNA Binding Domains (dsRBD) in the classical α-β-β-β-α conformation linked by a flexible 20 amino acid sequence (Nanduri et al., 1998). It also harbours a third basic region essential for PKR dimerization and autophosphorylation. This domain is followed by the C-terminal catalytic serine/threonine kinase domain (Heinicke et al., 2009, Lemaire et al., 2008, Dey et al., 2005). The linear protein structure of PKR and its interaction domains with other proteins are described **in Figure 3.**

Figure 3. The structure of the Protein Kinase R



(Okumura et al., 2013)

PKR induction and activation by Interferon and dsRNA

PKR is induced by IFN and is mainly activated by dsRNA. PKR's transcription is highly enhanced in the presence of IFN which classifies it as an IFN Stimulated Gene (ISG). IFNs are produced in the presence of viral, bacterial, parasitic and fungal infections but also by certain tumours. IFNs are cytokines which can signal in an autocrine or paracrine manner to neighbouring cells to activate antiproliferative and antiviral responses. When IFN binds to its dimeric IFN receptor, it triggers a signalling cascade through the JAK/STAT pathway which leads to the activation of promoters containing an IFN-Stimulated Response Element (ISRE) (De Andrea et al., 2002). The PKR gene EIF2AK2 contains an ISRE and is therefore an ISG (Ward and Samuel, 2002).

Once PKR binds to dsRNA, it dimerises and autophosphorylates on Threonines 446 and 451 (Dey et al., 2005). Other modifications are required for PKR activation, such as Small Ubiquitin-like Modifiers (SUMO), called SUMOylations, on lysines 60, 150 and 440 (de la Cruz-Herrera et al., 2014). Many viruses rely on dsRNA for their replication inside the cell. If the presence of these viral dsRNA is sufficient for maintaining PKR's activation, PKR will contribute to the repression of the viral replication (Sadler and Williams, 2007). The influenza B virus genomic RNA activates PKR and contributes to the attenuated virulence of this viral strain (Dauber et al., 2009). However, in the case of HIV-1, the TAR RNA only induces a transient activation of PKR, which is not sufficient to carry out a prolonged antiviral function (Clerzius et al., 2009).

Other activators of PKR include the cellular protein PKR Activator (PACT), and its ortholog RAX in mice (Patel and Sen, 1998, Ito et al., 1999). Proteins ISG15 and the tumour

Melanoma Differentiation Associated suppressor 7 (MDA-7) also activate PKR (Okumura et al., 2013, Pataer et al., 2005). Finally, some other factors can induce the activation of PKR such as heparin, an anticoagulant naturally produced by basophils and mast cells, or some cytokine mRNAs (Fasciano et al., 2005).

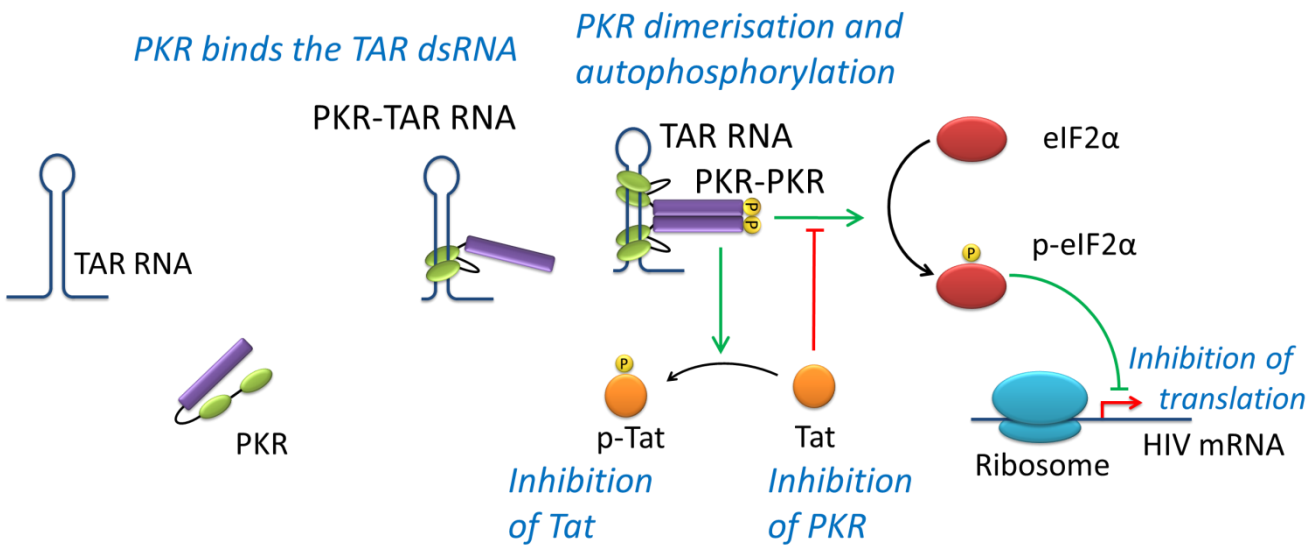
Modulation of eIF-2α phosphorylation by the HIV-1 Tat protein

PKR's main protein target is the eIF-2α. Activated PKR phosphorylates eIF-2α on its serine 51 which blocks the factor eIF-2B from cycling the ternary eIF2-GTP-Met-tRNAi required for protein synthesis. This inhibits the loading of the ribosome onto mRNAs and stops translation of cellular and viral mRNAs (Langland et al., 2006).

This block can be partially alleviated by the viral protein Tat by several mechanisms. Tat sequesters the HIV TAR RNA preventing the activation of PKR. Tat also interacts with PKR through a sequence comprised between its amino acids 40 to 58. Tat acts as a competitive inhibitor of eIF-2α due to a sequence homology with eIF-2α (Cai et al., 2000, Clerzius et al., 2011, Bannwarth and Gatignol, 2005). Furthermore, Tat is phosphorylated by activated PKR on three residues (S62, T64 and S68), thereby enhancing Tat RNA binding properties (Endo-Munoz et al., 2005). Another study suggests that phosphorylation of Tat by PKR on residues T23, T40, S46, S62 and S68 inhibits Tat's activity at different levels such as Tat nuclear localisation, TAR RNA binding and Cyclin T1 recruitment (Yoon et al., 2015).

PKR also phosphorylates other targets when activated, such as the tumour suppressor p53 (Bennett et al., 2012), the RNA helicase A (RHA) (Sadler et al., 2009), B-56α (Ruvolo et al., 2008), ILF-2/NF90 (Parker et al., 2001), or Cell division cycle 2 protein (Cdc2) (Yoon et al., 2010a). PKR activation and counteraction by Tat during HIV-1 infection are summarised in **Figure 4.**

Figure 4. PKR activation and counteraction by Tat during HIV-1 infection



PKR binds the double stranded HIV TAR RNA through its two dsRBDs. PKR is then activated by homodimerisation and autophosphorylation. Activated PKR phosphorylates eIF2α which inhibits translation of viral and cellular mRNAs. HIV protein Tat inhibits PKR kinase activity by acting as a competitive inhibitor. Multiple phosphorylation of Tat inhibits its transactivation activity by inhibiting its nuclear localisation, its binding to the TAR RNA and its recruitment of CycT1 and CDK9.

Cellular proteins contributing to PKR regulation: ADAR1, TRBP and PACT

ADAR1: ADAR1 is an Adenosine Deaminase Acting on RNA. Its substrate is the dsRNA and it converts Adenines into Inosines. Inosines are then recognised by the cellular translational machinery as Guanosines, thereby inducing changes in the protein sequences (Nishikura, 2010). ADAR1 is present in three isoforms; ADAR1 p150 is the IFN inducible cytoplasmic form of the protein and the p110 and p80 are the constitutively active nuclear forms. The p150 isoform possesses two Z DNA Binding Domains (ZDBD) at the N-terminus, three dsRBDs and a catalytic deaminase domain at the C terminus. The p110 isoforms lack the first ZDBD and p80 lacks the ZDBDs and the first dsRBD (Clerzius et al., 2009).

TRBP: The TAR RNA Biding Protein (TRBP) cDNA was first identified due to the TAR RNA binding properties of the protein it encoded (Gatignol et al., 1991). TRBP contains two dsRBDs and a medipal domain which is the interaction domain with the proteins DICER, Merlin and PACT (Daniels and Gatignol, 2012). TRBP is part of the RNA-Induced Silencing Complex (RISC) which is responsible for the processing of pre-microRNAs (pre-miRNA) into mature miRNAs. Micro RNAs mediate the silencing of their target mRNAs, therefore post transcriptionally controlling the expression of target proteins (Redfern et al., 2013). TRBP strongly interacts with the HIV-1 TAR RNA through its second dsRBDs, which is rich in Arginine and Lysine residues (Daviet et al., 2000, Erard et al., 1998, Gatignol et al., 1993).

PACT: The Protein kinase R ACTivator (PACT) was first identified in a two-hybrid assay using a non-catalytically active PKR as bait. Kinase assays revealed that PACT and its murine ortholog RAX were capable of activating PKR in the absence of dsRNA (Patel and Sen, 1998, Ito et al., 1999). PACT is a 313 amino acid long protein with two dsRBDs and a C-terminal PKR activation domain. PACT has a very similar structure to TRBP and in a resting cell the two proteins are bound through their dsRBDs and C-terminal domains (Laraki et al., 2008). In the presence of an oxidative stress, PACT is released and its Serines 18, 246 and 287 become phosphorylated (Huang et al., 2002, Gupta et al., 2003, Peters et al., 2001, Daher et al., 2009) and can then activate PKR. PACT is also a member of the RISC complex (Lee et al., 2006).

3- Modulation of PKR activation during HIV-1 infection

The PKR activation and inhibition during HIV replication

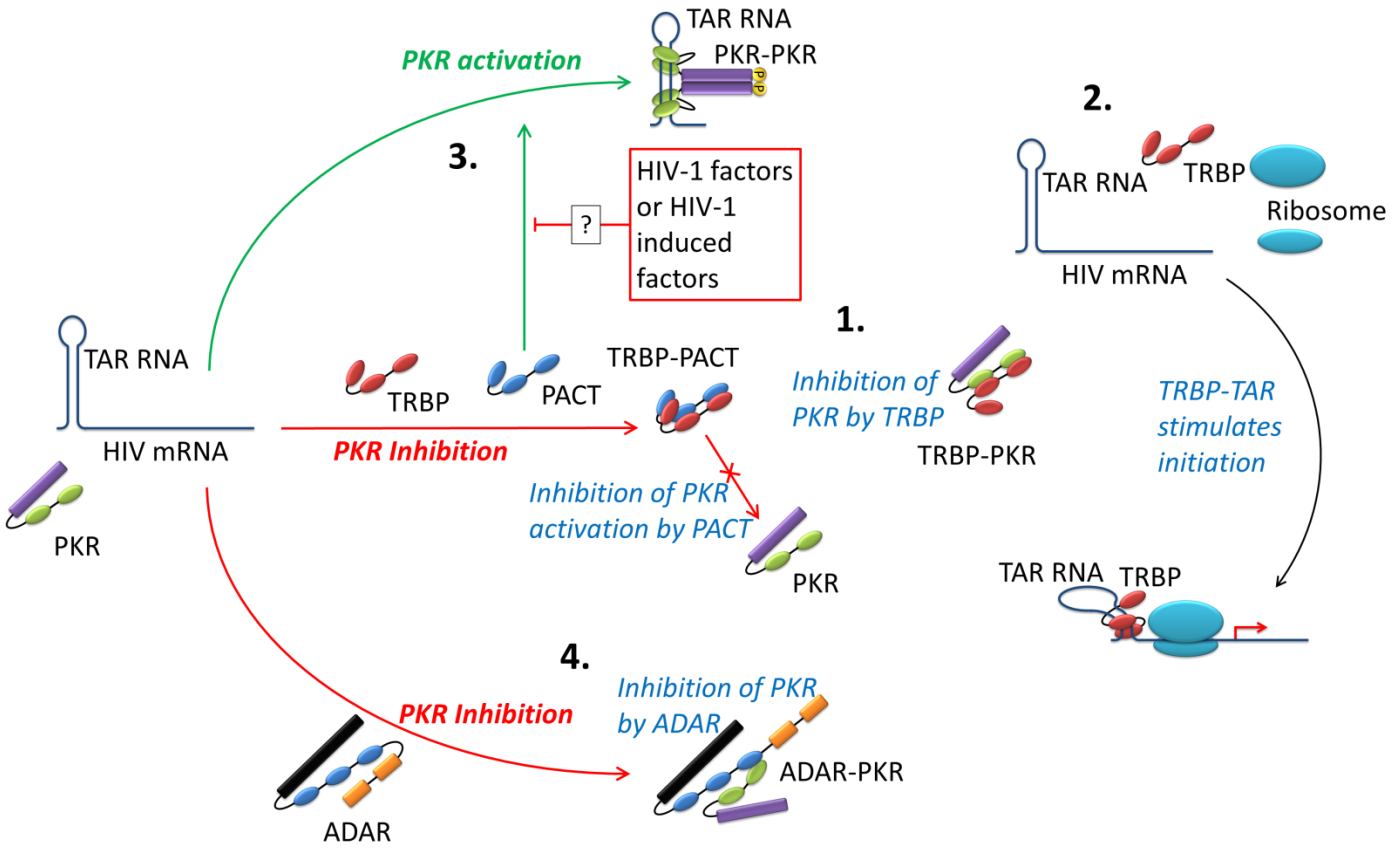
During HIV-1 infection of PBMCs, PKR is transiently activated at the beginning of infection and then inhibited during viral replication (Clerzius et al., 2009, Clerzius et al., 2013). Indeed, the initial low levels of TAR RNA likely activate PKR whereas high levels of TAR prevents its activation (Heinicke et al., 2009, Bannwarth and Gatignol, 2005).

In cell culture, overexpression of PKR has been shown to downregulate the expression of HIV (Adelson et al., 1999, Benkirane et al., 1997, Daher et al., 2001, Dimitrova et al., 2005, Muto et al., 1999) and the inhibition of PKR increases the viral production (Ong et al., 2005)

Interactions with ADAR, TRBP and PACT during HIV infection

During HIV-1 infection, the virus is able to replicate in permissive cells such as Lymphocytes and Monocytes/Macrophages, this implies that many mechanisms are involved in the repression of PKR's activity and several have been elucidated. Inhibition of PKR is achieved by interaction with proteins such as ADAR1, TRBP and the PKR activator PACT, which is converted into a PKR inhibitor during HIV infection. The interaction pathway of PKR inhibition by cellular factors during HIV replication is described in **Figure 5.**

Figure 5. Inhibition of PKR activation by cellular factors during HIV replication



PKR is transiently activated at the start of HIV infection and is inhibited at its peak via different mechanisms:

1. PKR is inhibited by direct interaction with TRBP. TRBP also inhibits PKR activation by sequestering the PKR activator PACT
2. TRBP promotes HIV mRNA translation by direct binding to the TAR RNA. This relaxes the structure of TAR and promotes the recruitment of initiation factors and binding of the ribosome
3. PKR is inhibited by direct interaction with ADAR1
4. PACT is converted into a PKR inhibitor during HIV active replication by unknown HIV factors or HIV induced cellular factors

ADAR1 is an ISG that has been linked to antiviral activity, through its RNA editing activity (Nishikura, 2010). However, it has also been shown that ADAR1 is a PKR inhibitor during many viral infections, promoting viral replication (Burugu et al., 2014, Clerzius et al., 2011, Gelinas et al., 2011, Pfaller et al., 2011, Samuel, 2011). The p150 and p110 isoforms of ADAR1 enhance the replication of several viruses such as Vesicular Stomatitis Virus (VSV) (Nie et al., 2007), HIV-1 (Clerzius et al., 2009, Doria et al, 2009) Measles virus (Toth et al., 2009),

Human T Lymphotropic Virus (HTLV) 1 and 2 (Cachat et al., 2014). ADAR1 expression is upregulated during HIV-1 infection in the Jurkat cell line and PBMCs (Clerzius et al., 2009, Clerzius et al., 2013). This upregulation occurs when HIV proteins are expressed, which correlates the activity of ADAR1 and the promotion of viral replication. ADAR1 is part of a protein complex, which includes TRBP and PACT and binds to PKR to prevent its activation. ADAR1 interacts with PKR through its first dsRBD (Clerzius et al., 2009).

TRBP promotes viral replication via several mechanisms. TRBP directly interacts with the TAR RNA allowing it to relax its structure and to promote the recruitment of initiation factors and of the ribosome. TRBP also inhibits PKR by direct binding through each of its dsRBDs, by sequestering the TAR RNA, therefore preventing detection by PKR and by directly interacting with PACT, preventing PACT from activating PKR (Burugu et al., 2014, Clerzius et al., 2011, Benkirane et al., 1997, Daher et al., 2009, Daniels and Gatignol, 2012, Dorin et al., 2003). TRBP also binds to the HIV RRE RNA blocking the ability of TRBP to bind small interfering RNAs (siRNAs) part of the RNA interference (RNAi) pathway. Some Adenoviruses are repressed by the RNAi pathway and the inhibition of RNAi by the RRE dependent inhibition of TRBP could also explain how HIV may similarly overcome a potential cellular RNAi block (Daniels et al., 2015).

PACT is the PKR activator in healthy cells. However, during HIV-1 infection PACT is converted into a PKR inhibitor. Although the mechanism of action of HIV or HIV induced cellular factors that operate this conversion is still unclear, PACT is part of the PKR inhibitory complex with ADAR1 and TRBP. Furthermore, PACT expression is stimulated during HIV infection, and PACT transfection in HEK293T cells stimulates viral production when co-transfected with an HIV molecular clone (Clerzius et al., 2013). Although this change in PACT activity on PKR could be due to the increased expression of ADAR1 a viral factor may also be involved in the mechanism (Clerzius et al., 2009, Cachat et al., 2014, Chukwurah et al., 2015)

The example of Astrocytes; Low levels of TRBP prevent HIV-1 replication

A good example of a cell type in which PKR acts as a restriction factor against HIV, is the astrocytic model. Astrocytes are glial cells present in the brain and the spinal cord. These cells can be infected by HIV but do not actively replicate the virus due to major blocks at the level of HIV protein synthesis, in the nuclear export of Rev and in the maturation of viral particles (Gorry et al., 2003, Gray et al., 2013). Astrocytes express low levels of TRBP due to the low levels of the transcription factor NF-Y in this cell type which is essential to TRBP transcription. The block in HIV protein synthesis can be attributed to the low levels of TRBP, which result in the activation of PKR and a block on translation. Overexpression of TRBP in astrocytes alleviated partially the block on HIV replication (Clerzius et al., 2009, Bannwarth et al., 2006, Ong et al., 2005, Bannwarth et al., 2001). In addition, the expression of siRNA or small hairpin RNAs (shRNAs) targeting TRBP can inhibit HIV replication in producing cells (Christensen et al., 2007, Eekels et al., 2011). This suggests that TRBP is one of the cellular mechanisms highjacked by HIV to target PKR and enhance its replication (Christensen et al., 2007, Eekels et al., 2011, Sanghvi and Steel, 2011). In summary, transient activation of PKR occurs at the beginning of the infection due to low levels of viral TAR RNA. The presence of high levels of TAR RNA during the active replication of HIV then contributes to PKR inhibition. Cellular mechanisms also contribute to PKR inhibition. The cellular protein TRBP inhibits PKR activation by direct binding and by competing with dsRNAs essential for PKR activation (Ong et al., 2005). Because of this, HIV is best adapted to replicate in cells with high levels of TRBP. HIV also promotes the transcription of ADAR1, another cellular inhibitor of PKR (Clerzius et al., 2009). This regulation represents an example of HIV's capability to highjack cellular mechanisms to counteract innate immunity and promote its replication. Finally HIV converts the

PKR activator PACT into a PKR inhibitor during its replication via HIV or HIV induced factors (Clerzius et al., 2013). The mechanism which converts PACT's activity is yet to be elucidated and is likely to involve several viral and cellular factors. In HIV producing cells, PKR is counteracted by the viral factor Tat but also by cellular factors TRBP, ADAR1 and PACT.

4- PKR, restriction factor of HIV?

Definition of a restriction factor

A restriction factor is a cellular component that inhibits viral replication. These cellular proteins have co-evolved with their viral target and are one of the first barriers of intrinsic immunity (Doyle et al., 2015, Malim and Bieniasz, 2012, Merindol and Berthoux, 2015, Simon et al., 2015). One of the key features of a restriction factor is the presence of positive selective pressure during their evolution. The evolutionary "arms race" between viruses and their hosts results in the fixation of non-silent mutations in the interaction domain between restriction factors and viral proteins. The fixation of mutations modifying the amino acid sequence of a given protein indicates the presence of a positive selective pressure applied on that protein. This positive selection is the consequence of two phenomena: 1) the necessity of the virus to replicate and 2) the role of the host's immune system to clear viral infections and evade inhibition by viral countermeasures.

Positive selection pressure can be measured by the ratio dN/dS, dN being the the non-silent mutations accumulated and dS the basal number of mutations or number of silent mutations fixed. A dN/dS ratio superior to 1 in one or several codons of a given gene proves the presence of a positive selection pressure on the corresponding amino acid(s). For a gene to be considered coding for a restriction factor it must answer to several criteria. 1) The gene must be present as orthologous genes in several species and the sequences considered must be correctly aligned. 2) The species considered must be organised in a phylogenetic tree. 3) These genes must present in their interaction domain with their viral substrate or inhibitors a ratio dN/dS > 1 (Johnson, 2013).

This genetic analysis has been carried out for several viral restriction factors such as APOBEC3G (Sawyer et al., 2004), Tetherin (Perez-Caballero et al., 2009), TRIM5 (Johnson and

Sawyer, 2009) and SAMHD1 (Laguette et al., 2012, Lim et al., 2012). In cells infected by HIV-1, APOBEC3G and Tetherin's activities are counteracted by viral protein Vif and Vpu, respectively and in cells infected by HIV-2, SAMHD1 is inhibited by the viral protein Vpx, allowing viral replication. One of the key features of intrinsic immunity is that it is constitutively active. However, all these genes coding for restriction factors are inducible by IFN and are established ISGs. Therefore, there is no strict barrier between intrinsic immunity and innate immunity and these restriction factors could be considered parts of both systems (Doyle et al., 2015).

PKR's antiviral function

It is well established that PKR overexpression inhibits the replication of several viruses but can PKR be considered a restriction factor? The notion of restriction factor has raised interest in HIV research after the discovery that endogenous cellular factors, such as TRIM5α, APOBEC3G, Tetherin and SAMHD1, are capable of restricting viral replication. However, these restriction factors become inactive in permissive cells due to viral accessory proteins that inhibit their activity (Doyle et al., 2015, Malim and Bieniasz, 2012, Merindol and Berthoux, 2015, Johnson, 2013). Other restriction factors prevent the replication of other viruses. Recent examples suggest that SAMD9 and WDR6 have an antiviral effect against the Vaccinia virus (Sivan et al., 2015), the promyelocytic leukaemia protein (PML) against the Dengue virus, the DEAD box helicase DDX60L against the hepatitis C virus (Grunvogel et al., 2015), Tetherin against the Hepatitis B virus (Yan et al., 2015) and TRIM32 against the influenza A virus (Fu et al., 2015). It is unknown which viral factors counteract these cellular proteins and the presence of a positive selective pressure during their evolution is yet to be demonstrated, which could result in them being classified as either restriction factors or resistance factors. Several HIV resistance factors have been identified such as MX2 and MxB (Goujon et al., 2013, Liu et al., 2013), the proteins

IFITM 1, 2 and 3 (Yu et al., 2015) and Schlafen 11 (Li et al., 2012). The mechanisms developed by HIV to evade these factors have not yet been discovered.

Early PKR studies demonstrated that PKR was capable of inhibiting several viruses after treating infected cells with IFN or after its overexpression. However, PKR was not classified as a resistance or restriction factor. During HIV infection, PKR is considered as part of the innate immunity as its expression and phosphorylation are induced by the viral infection (Clerzius et al., 2009, Clerzius et al., 2013). As every established restriction factor against HIV are also induced by IFN and have been shown to be under positive selective pressure, it would be of interest to determine if PKR is subjected to such positive selection.

Positive Selection and viral countermeasures

Two phylogenetic studies have demonstrated the relationship between different orthologs of PKR in vertebrates including primates (Elde et al., 2009, Rothenburg et al., 2009). The authors demonstrated the presence of amino acids under positive selective pressure in the interaction domain of PKR and its *Poxviridae* viral inhibitor, K3L. K3L is a protein found in several members of the *Poxviridae* and has an inhibitory effect on PKR and stimulates viral replication. The K3L interaction domain resembles eIF-2α and K3L acts as a competitive inhibitor of PKR (Langland et al., 2006, Elde et al., 2009). This positive selection only exists for PKR but not for other eIF-2α kinases such as HRI (response to haeme starvation), PERK (response to endoplasmic reticulum stress) and CGN2 (response to amino acid starvation) and these proteins have not been associated to antiviral functions (Rothenburg et al., 2009).

Similarly, a positive selective pressure was detected in the PKR binding domain that interacts with E3L, another *Poxviridae* protein that inhibits the dimerization and activation of PKR by direct interaction. This study shows the accumulation of non-silent mutations (dN/dS >

1) in the PKR kinase domain, compared to other eIF-2α kinases, proving the presence of positive selection imposed by K3L and E3L (Rothenburg et al., 2009).

HIV restriction by PKR and viral countermeasures

The two previous studies demonstrated that PKR can be considered as a restriction factor of the *Poxviridae* family and that these viruses have evolved countermeasures such as the viral proteins K3L and E3L (Elde et al., 2009, Rothenburg et al., 2009). The HIV-1 genome codes for a regulatory protein called Tat, which has a similar function as K3L. Tat acts as a competitive inhibitor, by binding to the kinase domain of PKR and inhibiting its binding and phosphorylation of eIF-2α (Cai et al., 2000). The interaction domain between Tat and PKR has not been precisely characterised; however, it is likely situated in the same domain of interaction with eIF-2α (kinase domain) and that the positive selective pressure observed for PKR in this domain for *Poxviridae* can also be attributed to HIV. The study of these interaction domains could shed light on PKR function as an HIV-1 restriction factor.

One major difference between the restriction factors TRIM5α, Tetherin, APOBEC3G, SAMHD1 and PKR is that PKR requires activation for its antiviral activity. When the factors listed above are constitutively active and become inactivated in the presence of viral inhibitors, PKR only requires its dephosphorylation or dephosphorylation of its target eIF-2α to become inactive and allow HIV replication.

Overall, PKR acts as a restriction factor on HIV replication. The positive selection of several amino acids in its catalytic kinase domain could be the reason why PKR has maintained its antiviral activity against HIV. Indeed, SIV crossed the species barrier to become HIV much more recently than the members of the Poxviridae.

The complexity of the mechanisms regulating restriction and resistance factors and their co-evolution, resulting in the ability of HIV to replicate in certain cell types is not entirely understood. It is likely that for HIV resistance factors such as MxB, IFITMs, and Schlafen 11, which do not have any specific viral countermeasures, are cellular inhibitors recruited or induced by HIV to counteract their antiviral activity and allow viral replication. PKR could serve as an example to better understand the evolution of these mechanisms and how viruses overcome cellular blocks on their replication. Furthermore, PKR and other restriction/resistance factors may contribute to HIV latency and the establishment of viral reservoirs.

5- HIV and Latency

Definition of latency and HIV latency

Viral latency refers to the dormant state certain viruses enter during their replication cycle within the cell. This part of the viral replication cycle is characterised by an arrest in production of viral particles. As a retrovirus, HIV-1 enters the cell and integrates its genome into the cell's chromosome with no or little expression of viral proteins (Perng and Jones, 2010). This allows the virus to remain undetected by the host's immune system for prolonged periods of time. Latency is a complex phenomenon and can be due to multiple factors such as the presence of a restriction factor targeting a crucial step of the replication cycle or the absence of a cellular factor required by the virus. The repressed viral genome can be reactivated by an external stimulus such as oxidative stress, chemical compounds, immune activation or sunlight which can modify the gene expression or the activation of some proteins. If the changes in the cell affect the latency inducing factors, the viral genome starts to be expressed and produces new viral particles. The reactivation of latent cells results in the infection of nearby cells and allows the virus to spread throughout the infected host or to other individuals. Latency can be classified in two main mechanisms; episomal and proviral latency.

Episomal latency occurs when the viral genetic material is organised into episomes while latent. In this category, the virus is stabilised in the cell cytoplasm or the cell nucleus independently from the cellular genetic material. This type of latency is less stable than proviral latency as is more likely to be detected by the cell intrinsic or innate immune sensors and to be degraded. The *Herpesviridae* rely on this type of latency allowing them to evade the immune response (Grinde, 2013).

The Herpes virus simplex 1 and 2 (HSV-1 and HSV-2) and Varicella Zoster virus (VZV) all latently infect neural ganglia (Decman et al., 2005, Margolis et al., 2007, Eshleman et al., 2011). Their genetic material is organised in an episome within the cell cytoplasm and when the

virus enters the latent stage it transcribes a Latency Associated Transcript (LAT) RNA. The LATs regulate the host gene expression and interfere with cell death mechanisms.

The herpes viruses Epstein-Barr Virus (EBV) and Kaposi's Sarcoma-associated Herpesvirus (KSHV) also rely on episomal latency during their life cycle. The main difference with HSV-1, HSV-2 and VZV is the nuclear localisation of their episome during latency and their cell tropism. EBV and KSHV preferentially infect B lymphocytes (Niller et al., 2011, Uppal et al., 2014).

Proviral latency refers to the integration of the viral genome into the host's genome without the active production of new viral particles. This type of latency presents many advantages for the virus one of them being the perpetuation of the integrated provirus in the progenitor cells. Furthermore, if the cell divides, the integrated provirus will also be replicated. This integration is a required step of the replication cycle in the *Retroviridae* family, the best studied example being HIV (Marcello, 2006, Bisgrove et al., 2005). HTLV-1 is another example of a virus using provirus latency in its replication cycle. Indeed, the HTLV-1 infection is latent in most cases and the infection is usually asymptomatic in patients but can lead to the development of malignancies in 3-5% of cases. It is thought that HTLV-1 spreads principally by mitotic expansion of infected cells and by low levels of reactivation and reinfection (Philip et al., 2014, Bangham et al., 2013).

HIV latency was first used to describe the long period during which HIV infection does not present any symptoms. The development of RT-PCR techniques allowed the detection of low levels of viral replication even during the asymptomatic phase of the infection, indicating that HIV was actively replicating even during the asymptomatic phase (Piatak et al., 1993). *In vitro* evidence of latency was first suggested when infected cell lines which showed low HIV viral replication could be stimulated by using T-cell activating agents (Folks et al., 1986). It was later determined that cell factors such as NF-κB were essential for HIV active replication and this factor is upregulated in active $CD4^+$ T cells (Duh et al., 1989). The main consequence and

clinical significance of HIV latency is the establishment of viral reservoirs in HIV infected active $CD4^+$ T cells, which later differentiate into resting memory cells (Siliciano and Greene, 2011). Athough HIV is known to establish reservoirs in different cell types, the main reservoir is represented by the long living resting $CD4^+$ memory T cells (Chun et al., 2015).

Approaches to curing HIV

Although HAART can repress HIV replication to undetectable levels and that HIV is now considered a chronic disease, there is currently no HIV cure. This is largely due to the persistence of HIV reservoirs and even prolonged HAART therapy cannot eradicate the virus present in latently infected cells (Finzi et al., 1997, Wong et al., 1997, Chun et al., 1997). Stopping HAART treatment would lead to a relapse and disease progression, with active viral replication that can be detected generally in two weeks after treatment interruption (Davey et al., 1999). The only case of HIV being cured has been the so called "Berlin patient". This HIV positive individual developed an acute myeloid leukemia (AML) and received a haematopoietic stem cell transplant from a donor harbouring a resistance mutation against HIV. This mutation, Δ32CCR5 is a mutation of the CCR5 receptor essential for viral entry. Clearing of the Hematopoietic reservoirs and presence of a resistance mutation appeared to be sufficient to control HIV infection (Hutter et al., 2009). The lack of less intrusive cure and the clear limitations involved with this therapeutic approach such as donor matching, and the rarity of the resistance mutation outlines the necessity for further research in that field. Recent findings from the French VISCONTI cohort show that 14 patients treated in early stages of HIV infection (acute phase) are characterised with very low viral reservoir and these patients were shown to control viremia, even years after interruption of antiretroviral therapy (Saez-Cirion et al., 2013). These findings suggest that if viral reservoirs are sufficiently controlled very early after the acute infection, patients could be functionally cured from HIV infection. However, these cases cannot be generalised as similar treatment

interruptions did not give rise to a functional cure but to only short remission in most patients, exemplified by the so called “Mississippi baby” (Luzuriaga et al., 2015). The longest on-going remission occurs in a French young woman after 12 years of treatment interruption (Frange et al., 2016).

There are currently two main approaches to developing an HIV cure; the “shock and kill” approach which aims at achieving a sterilising cure, and the induction of “deep latency” which aims at developing a functional cure (Dahabieh et al., 2015). The sterilising cure approach aims at reactivating the latent reservoirs which would then be detected and cleared by a specific killing of the reactivated cells or by an extremely active immune system. The functional cure, also called long term remission or deep-latency, consists in maintaining the virus in an irreversible latent state, even in the absence of HAART (Deeks et al., 2016).

Reactivation from latency and Latency Reversing Agents (LRAs)

One of the conditions for achieving a sterilising cure is the reactivation of reservoirs from their latent state. Latency Reversing Agents or LRAs are chemical compounds capable of reactivating HIV from latency. The reactivated cells could then be cleared by cytotoxic T lymphocytes (CTLs) by intensifying HAART or by other means to be developed. There are several classes of LRAs acting on different latency inducing mechanisms (Xing and Siliciano, 2013).

Histone Deacetylase inhibitors (HDACi): Histone cores are made of proteins involved in DNA packaging. Histone acetylation by histone acetyltransferases (HATs) is associated with actively transcribed genomic regions. Histone deacetylases are usually associated with transcriptional repression. During latency, HDACs are recruited to the HIV provirus and prevent its transcription. HDAC inhibitors prevent the deacetylation of histone cores and result in HIV reactivation (Ylisastigui et al., 2004, Matalon et al., 2011). HDACi include compounds such as

suberoylanilide hydroxamic acid (SAHA or vorinostat) (Contreras et al., 2009) and givinostat (ITF2357), Panobinostat and Romidepsin (Matalon et al., 2010).

Histone Methyltransferase inhibitors (HMTi): Histone methylation can either increase or decrease the transcription of genes depending on how many methyl groups are added and which amino acids of the histone are methylated. Some Histone Methyltransferases (HMTs) such EZH2, SUV39H1 and G9a have been reported to inhibit HIV translation (du Chene et al., 2007, Friedman et al., 2011, Imai et al., 2010). Some HMTi have been reported to have latency reversing properties such as BIX01294, a specific G9a inhibitor (Imai et al., 2010) and Chaetocin an Suv39H1 inhibitor (Bouchat et al., 2012).

DNA Methyltransferase inhibitors (DNMTi) Although DNA methylation implication in HIV latency is still controversial, some DNMTi have synergistic activity with PKC agonists such as prostratin in cell culture (Fernandez and Zeichner, 2010). Decitabine (5-aza-2' deoxycytidine, aza-CdR) and its analog azacitidine (5-azacytidine, Vidaza®) are both examples of DNMTi and are FDA approved molecules for the treatment of myelodysplastic syndrome (Fenaux, 2005).

Protein Kinase C activators: The Protein Kinase C (PKC) is an activator of the NF-κB and AP-1 signalling pathways which in turn upregulate HIV-1 expression. PKC agonists are potent HIV LRAs and act by recruiting PKC from the cytoplasm to the cell membrane (Marquez et al., 2008). PMA and 12-deoxyphorbol-13-acetate (prostratin) are both phorbol esters and the most well established PKC agonists (Williams et al., 2004, Kulkosky et al., 2001). Bryostatin is a macrolide lactone that is also known to activate the PKC pathway and can be used as an LRA (Perez et al., 2010)

Positive Transcription Elongation Factor b (P-TEFb): P-TEFb is a transcription elongation factor acting with RNA polymerase II activity to enhance transcriptional elongation. It is composed of cyclinT1 and CDK9 in which CDK9 mediates the hyperphosphorylation of RNA

pol II. HIV relies on RNA Pol II for transcription of its genome and the HIV Tat protein binds to cyclin T1 to recruit CDK9 and increase transcriptional elongation (Gatignol, 2007). Disrupting P-TEFb Tat interaction can induce HIV reactivation by liberating Tat activity and increasing transcriptional elongation. Hexamethylene bisacetamide (HMBA) is a P-TEFb activator and acts through the PI3K/Akt pathway (Contreras et al., 2007, Choudhary et al., 2008).

Other types of LRAs that do not belong to the classes listed above have been suggested such as disulfiram, an FDA approved drug used in the treatment of alcoholism or cocaine addiction, which does not activate T cells (Xing et al., 2011). Targeting new latency inducing mechanisms could help to identify new LRAs in the future. Finally, it is important to note that most LRAs are more effective when used in combination and many recent studies focus on determining which combinations work best (Darcis et al., 2015, Darcis et al., 2016).

6- The role of the p53 pathway during HIV replication

The p53 pathway

The tumour suppressor p53 is a transcription factor encoded by the human gene TP53. There are 12 known p53 isoforms produced across different cell types through alternative translation initiation, promoter usage and splicing (Surget et al., 2013). Differential expression of p53 isoforms or mutations of the TP53 gene have been linked to numerous malignancies and p53 profile has been shown to influence response to treatments. p53 has been shown to control cancer formation via 2 different pathways; it can promote cell cycle arrest to allow DNA repair machinery to operate or it can activate proapoptotic genes and initiate cell death (Khoury and Bourdon, 2011, Zilfou and Lowe, 2009).

p53 can be activated by various cellular stresses such as DNA damage, ultra violet light, ionising radiations, ribonucleotide starvation or oxidative stress. When inactive, p53 is bound to its inhibitor mdm2, which sequesters p53 and targets it to the proteasome by ubiquitination and inhibits its nuclear localisation. After activation by stress, p53 becomes phosphorylated by various kinases involved in genome integrity checkpoint such as ATM–Rad3-related protein (ATR), Ataxia Telangiectasia Mutated (ATM), Checkpoint kinase 1 (CHK1), Checkpoint kinase 2 (CHK2) or by kinases of the Mitogen-activated protein kinases (MAPK) family which respond to stress such as c-Jun N-terminal kinases (JNK1-3), extracellular signal–regulated kinases (ERK1-2) and p38 MAPK. Phosphorylation stabilises p53 and allows it to dissociate from mdm2 thus increasing p53 levels within the cell. p53 then translocates to the nucleus where it acts as a transcription factor in a tetrameric conformation. If DNA repair occurs, the cell cycle is allowed to restart. If the DNA damage is not resolved, p53 will activate proapoptotic genes such as Bax, Puma, Scotin and Fas and repress antiapoptotic genes such as Bcl2 leading to cell death (Oren, 2003, Surget et al., 2013, Khoury and Bourdon, 2011).

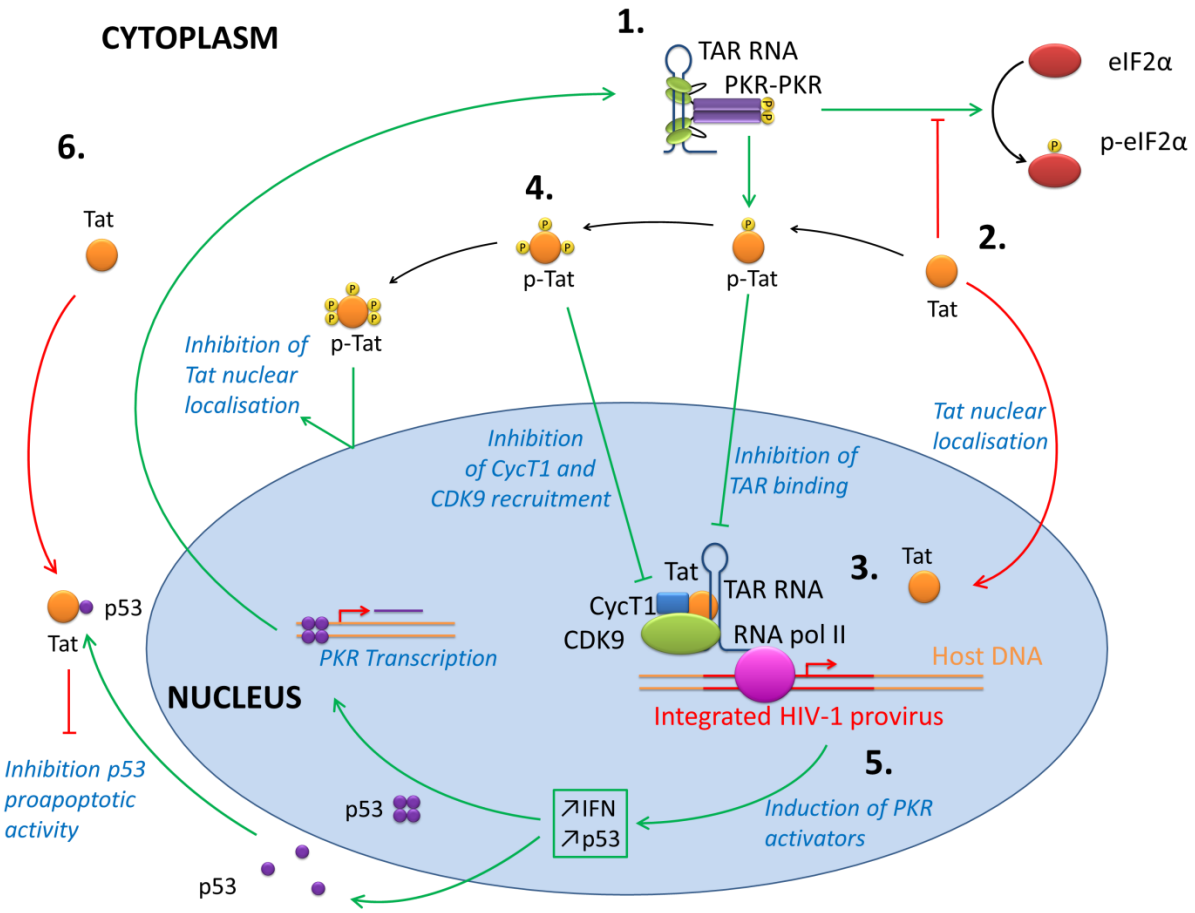
Interplay between the PKR and p53 pathways

p53 can induce PKR expression via its cis-acting element which is separated from the Interferon-stimulated responsive element (ISRE) in the PKR promoter. Activation of p53 significantly increases PKR expression and promote the activation of proapoptotic pathways (Yoon et al., 2009). Inversely, PKR activates p53 by promoting its sumoylation. The RAX/PACT-dependent activation of PKR promotes the association of PKR with the SUMO E2 ligase Ubc9 which sumoylates p53 on Lysine 386 (Bennett et al., 2012). PKR also directly associates with p53 and phosphorylates it on Serine 392 *in vitro* (Cuddihy et al., 1999). In addition dsRNA could be an activator of the p53 pathway independently from the IFN pathway (Hummer et al., 2001). Although the interplay between the two pathways clearly exists, further investigation of the mechanisms of action is still required (Garcia et al., 2007, Dabo and Meurs, 2012, Yoon et al., 2009).

The role of p53 during HIV infection

Yoon et al. (2015) recently demonstrated that p53 restriction of HIV could be attributed to PKR by multiple phosphorylations of viral protein Tat. The study shows that p53 silencing leads to increased viral production in infected cells. Reintroduction of p53 could suppress replication which was in turn enhanced by PKR silencing. PKR transcription was shown to be promoted by p53 in a previous study (Yoon et al., 2009). Yoon et al. (2015) demonstrate that p53 is activated by HIV-1 followed by an increase in PKR transcription and activation. PKR interacts directly with Tat and phosphorylates it on five residues (T23, T40, S46, S62 and S68). Differential phosphorylation levels inhibit three distinct Tat functions: 1) Tat-TAR RNA binding, 2) Tat ability to recruit cyclin T1 and Cyclin-Dependent Kinase 9 (CDK9), and Tat nuclear localisation. The restriction pathway of HIV-1 by PKR and p53 is described in **Figure 6.**

Figure 6. HIV-1 restriction by PKR and p53 and Tat counteraction



1. PKR is activated by the double stranded TAR RNA and Phosphorylates eIF2α
2. Tat acts as a competitive inhibitor of PKR. Tat phosphorylation inhibits eIF2α activation by binding to the kinase domain of PKR.
3. Tat is expressed during HIV replication and translocates to the nucleus. Tat binds TAR when HIV mRNA is transcribed and recruits CycT1 and CDK9 (pTEFb). pTEFb hyperphosphorylates the RNA pol II. Recruitment of Tat and pTEFb to TAR releases a translational block and stimulates the expression of the HIV genome.
4. Tat activity is also inhibited by PKR phosphorylation. PKR phosphorylates Tat at multiple sites and inhibits its ability to bind to TAR, to recruit CycT1 and CDK9 and to relocate to the nucleus.
5. The expression of the integrated HIV genome induces the production and activation of PKR activators such as IFN and p53. High levels of activated p53 and IFN stimulate the expression of PKR.
6. Direct interaction between Tat and p53 and inhibition of p53 proapoptotic signalling

Modified from (Yoon et al., 2015)

Inhibition of p53 by Tat during HIV infection

Several studies have shown that HIV Tat inhibits p53, which contributes to the productive viral replication. Tat binds directly to p53 through its amino acids 1-35 and 47-57 and blocks p53 activity and its downstream effects in its proapoptotic pathway. Therefore, a complex interplay exists between p53, PKR and Tat during HIV replication, but the intimate mechanisms and the role of p53 during the viral replication remains to be determined.

HYPOTHESIS, OBJECTIVES AND EXPERIMENTAL APPROACHES

Hypothesis

PKR activation and inhibition of translation initiation is a contributing factor in the establishment of HIV latency in specific cell types through different pathways and can be targeted for the development of new therapies.

Objectives

Project 1

- Demonstrate the inhibitory effect of PKR on HIV-1 replication
- Demonstrate the inhibitory effect of TRBP, PACT and ADAR1 on PKR activity

Project 2:

- Identify the effects of established LRAs on the PKR pathway
- Inhibiting PKR to reactivate an HIV-1 latency model
- Identify new Latency Reversing Agents (LRAs) that target PKR.
- Test the efficacy of PKR inhibitors as LRAs in latently infected PBMCs

Project 3

- Characterise the role of p53 in HIV replication
- Characterise the interplay between the p53 and PKR pathways during HIV replication

Experimental approaches

Project 1:

- Transfecting the HEK293T cell line with a Luciferase reporter gene under the control of the HIV promoter (LTR) and study the effect of PKR and PKR partners on viral replication by co-transfecting cells with plasmids expressing PKR, TRBP, ADAR and PACT. Assess the expression of the HIV promoter by Luciferase assay.
- Transfecting the HEK293T cell line with an HIV molecular clone and study the effect of PKR and PKR partners on viral replication by co-transfecting cells with plasmids expressing PKR, TRBP, ADAR and PACT. Assess the effect on the PKR pathway by Western Blot

Project 2:

- Reactivate an HIV latent model with LRAs and study the effect on the PKR pathway by Western Blot.
- Reactivate the HIV latent model with LRAs and sort by FACS the treated-reactivated and treated-non-reactivated cells and study the effect on the PKR pathway.
- Use PKR inhibitors (Imoxin, and Sunitinib) and assess the reactivation pattern in the latent model by FACS and Western blot

Project 3:

- Transfect two cell lines: HCT116 p53wt and HCT 116 p53KO with HIV molecular clones and assess the viral production by Reverse Transcriptase assay.

MATERIALS AND METHODS

Cell culture and transfections

HEK293T (ATCC CRL-11268) and HCT116 p53wt and HCT116 p53KO cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). CEM T4 GagZipGFP and THP-1 GagZipGFP cell lines were maintained in RPMI-1640 (Invitrogen) with identical supplementation.

For transfection of HEK293T cells with plasmids and HIV molecular clone pNL4-3, cells were plated in six-well plates at 75% confluence, 24 h prior to transfection using polyethylenimine (PEI) following manufacturer's protocol (Polysciences). For transfection of HCT116 cells with pNL4-3, INDIE, AD8 and BlueScript (1 μg/well in a 6-well plate) was then transfected using TransIT-LT (Mirus) 24 h after plating. Supernatants and lysates were then collected 48 h after transfection of pNL4-3.

Transfection of HIV-1 molecular clones and RT assay

HEK 293T were transfected as above with pNL4-3 molecular clone. Cell supernatants were collected 48 h post transfection and viral production assayed for standard RT assay. Supernatants from transfected HEK 293T cells were used for infection of Jurkat or PBMCs.

The supernatant of transfected HEK 293T and HeLa cells were analyzed for virus production by RT assay after 48h incubation. 5 μl of viral supernatant in 50 μl of supplemented RT cocktail (60 mM Tris–HCl, 75 mM KCl, 5 mM MgCl 2, 1.04 mM EDTA, 1 % Nonidet P-40) was incubated

at 37°C for 2 h. 5 µl of each reaction mixture were spotted onto DEAE filter paper (Whatman). The membranes were washed and counted as described in Scarborough et al. (2014).

Reactivation of the CEM T4 GagZipGFP and THP1 GagZipGFP cells

HIV-1 CEM T4 GagZipGFP and THP1 GagZipGFP cell lines were designed by Dr. Alan Cochrane's laboratory (Department of Molecular Genetics, University of Toronto) and was based on a latent model developed by Das et al. (2004) with a Tet-on/Tet-off system dependent on doxycycline and a GFP reporter. Cells were treated with 200ng.µL^{-1} of doxycycline and either 400ng.mL^{-1} of prostratin or 1.1µg.mL^{-1} of SAHA (vorinostat) for 48 h. Cells were then prepared for flow cytometry analysis by resuspending them in PBS 3% FBS 1% EDTA and filtered through 40µm Corning cell strainers (CEM T4 cell line) and 70µm (THP-1 cell line).

Cells were then sorted by flow cytometry (BD FACS Aria Fusion) and separated using GFP as the selection marker. Data were acquired with BD FACS Diva and analysed with Flowjo software.

Luciferase Assays

Cells were transfected as described above. After 48 h, the culture media was removed and cells were washed with PBS 3 times. Cells were lysed with 200µL of Luciferase Cell Culture Lysis Reagent (LCCLR) (Promega Luciferase kit) and incubated at room temperature for 15 min. Cell lysates were cleared of debris by centrifugation. 50µL of Luciferase Assay Reagent (Promega) were added to 10µL of cell lysate and acquired with a Glomax luminometer (Promega).

Immunoblotting

Cellular and viral lysates (50 to 90 μg) were boiled for 5 min at 95°C in SDS loading buffer (0.5 M Tris HCl, 25% SDS, 20% Glycerol and 0.01 % Bromophenol blue). Samples were loaded into a 10% SDS PAGE gel. The proteins were then transferred onto a nitrocellulose membrane by semi-dry transfer (Bio-Rad Laboratories) for 1 hour at 10V with transfer buffer (48mM Tris-HCl, 39mM glycine, 0.375% SDS and 20% ethanol). The membranes were then blocked in 5% milk diluted in Tris saline buffer 1% Tween20 (TBS-T) for 1 hour. The membrane was then washed 2 time with TBS-T and were incubated overnight at 4°C in primary antibodies (TBS-T, 3% BSA). Following the overnight incubation, membranes were washed 4 times in TBS-T, and incubated with TBS-T, 3% milk and secondary antibodies for 1 h. Membranes were washed again 4 times with TBS-T and Enhanced Chemiluminescence (ECL) solution for revealing.

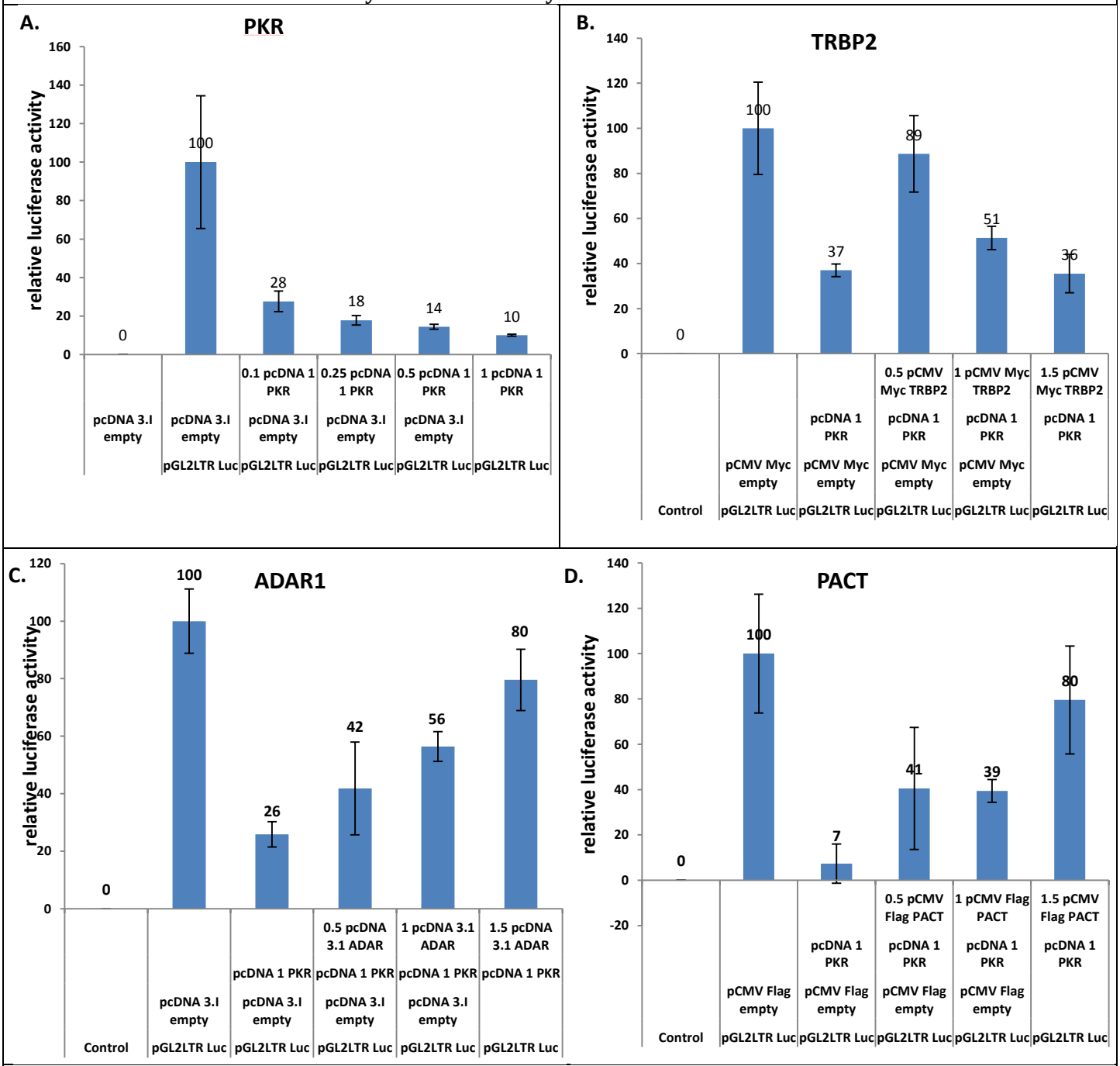
RESULTS

Project 1 – Inhibition of viral replication by the PKR pathway and inhibition of PKR by ADAR1, TRBP and PACT

Inhibition of the HIV LTR promoter by PKR and reversion by ADAR1, TRBP and PACT in HEK 293T

Our laboratory previously showed that overexpressing PKR could inhibit the expression of the HIV promoter LTR (Clerzius et al., 2009). LTR expression can be rescued when PKR is overexpressed if the proteins ADAR1, TRBP and PACT are also overexpressed. These experiments were set to determine the antiviral function of PKR on the expression of the LTR and the inhibitory effect of ADAR1, TRBP and PACT and PKR activity. We used a plasmid containing the luciferase gene under the control of the HIV-1 LTR and plasmids expressing PKR, ADAR1, TRBP and PACT which were transfected in the HEK 293T cell line.

Figure 7. Inhibitory effect of PKR on HIV-1 LTR expression in HEK 293T, counteracted by TRBP, PACT and ADAR1 determined by Luciferase assay.



Transfection of HEK 293T cells with **A.** 0.05µg of a plasmid (pGLTR Luc) expressing the Luciferase gene under the control of the HIV-1 promoter (LTR) and increasing doses (0.1µg, 0.25µg, 0.5µg, 1µg) of a plasmid expressing PKR (pcDNA 1 PKR) **B.** 0.05µg of a plasmid (pGLTR Luc) expressing the Luciferase gene under the control of the HIV-1 LTR, 0.25µg of pcDNA 1 PKR and incremental doses (0.5µg, 1µg, 1.5µg) of a plasmid expressing TRBP2 (pCMV Myc TRBP2) **C.** 0.05µg of a plasmid (pGLTR Luc) expressing the Luciferase gene under the control of the HIV-1 LTR, 0.25µg of pcDNA 1 PKR and incremental doses (0.5µg, 1µg, 1.5µg) of a plasmid expressing ADAR1 (pDNA 3.1 ADAR) **D.** 0.05µg of a plasmid (pGLTR Luc) expressing the Luciferase gene under the control of the HIV-1 LTR, 0.25µg of pcDNA 1 PKR and incremental doses (0.5µg, 1µg, 1.5µg) of a plasmid expressing PACT (pCMV Flag PACT). In **A. B. C.** and **D.** 50µL of Luciferase Assay Reagent (Promega) were added to 10µL of cell lysate and acquired with a Glomax luminometer (Promega). Measurements were normalised to the controls.

In the experiment presented in **Figure 7.** the HEK 293T cell line was used to demonstrate the inhibitory effect of PKR on the expression of the HIV promoter (LTR) and how PKR interacting protein TRBP2, ADAR1 and PACT can counteract PKR activity when overexpressed. To monitor the expression of the HIV promoter, a plasmid (pGLTR Luc) expressing the Luciferase gene under the control of the HIV-1 promoter (LTR) was transfected into the cells. The value of the luciferase reading was used as the basal expression of the LTR in HEK 293T cells. Incremental doses of a plasmid expressing PKR (pcDNA 1 PKR) were then transfected to determine a plasmid quantity to transfect to obtain an 80% inhibition of the basal luciferase expression which was determined to be at 0.25µg. This 80% inhibition was then used to investigate the counteraction of PKR-induced HIV LTR inhibition by TRBP, ADAR1 and PACT. A consistent 80% inhibition of PKR in experiments 7B (63%), 7C (74%) and 7D (93%) due to experimental errors and to the large variations in values during luciferase assay as the reagents are very sensitive. These experiments were retained as the inhibition obtained was superior to 50% in every case and illustrated the inhibitory effect of TRBP2, ADAR1 and PACT on PKR in each case. In the Figure 7B, 7C and 7D, cells were transfected with 0.05µg of pGLTR Luc to determine the basal expression of the Luciferase. Cells were then transfected with incremental doses of plasmids expressing TRBP (pCMV Myc TRBP2), ADAR1 (pDNA 3.1 ADAR) and PACT (pCMV Flag PACT). For TRBP the doses allowed a recuperation of the expression of the LTR with a decrease in expression passed the threshold of 0.5µg of plasmid transfected. Both ADAR1 and PACT allowed a dose dependent recuperation of the expression of the LTR. PACT is a PKR activator but acts a s a PKR inhibitor in cells with a high amount of TRBP by forming a TRBP-PACT heterodimers (Laraki et al., 2008, Daher et al., 2009) it is also converted into an HIV inhibitor in HIV expressing cells (Clerzius et al., 2013). HEK293T cells readily express TRBP2 which explains the significant inhibition of PKR by PACT observed in panel 7D.

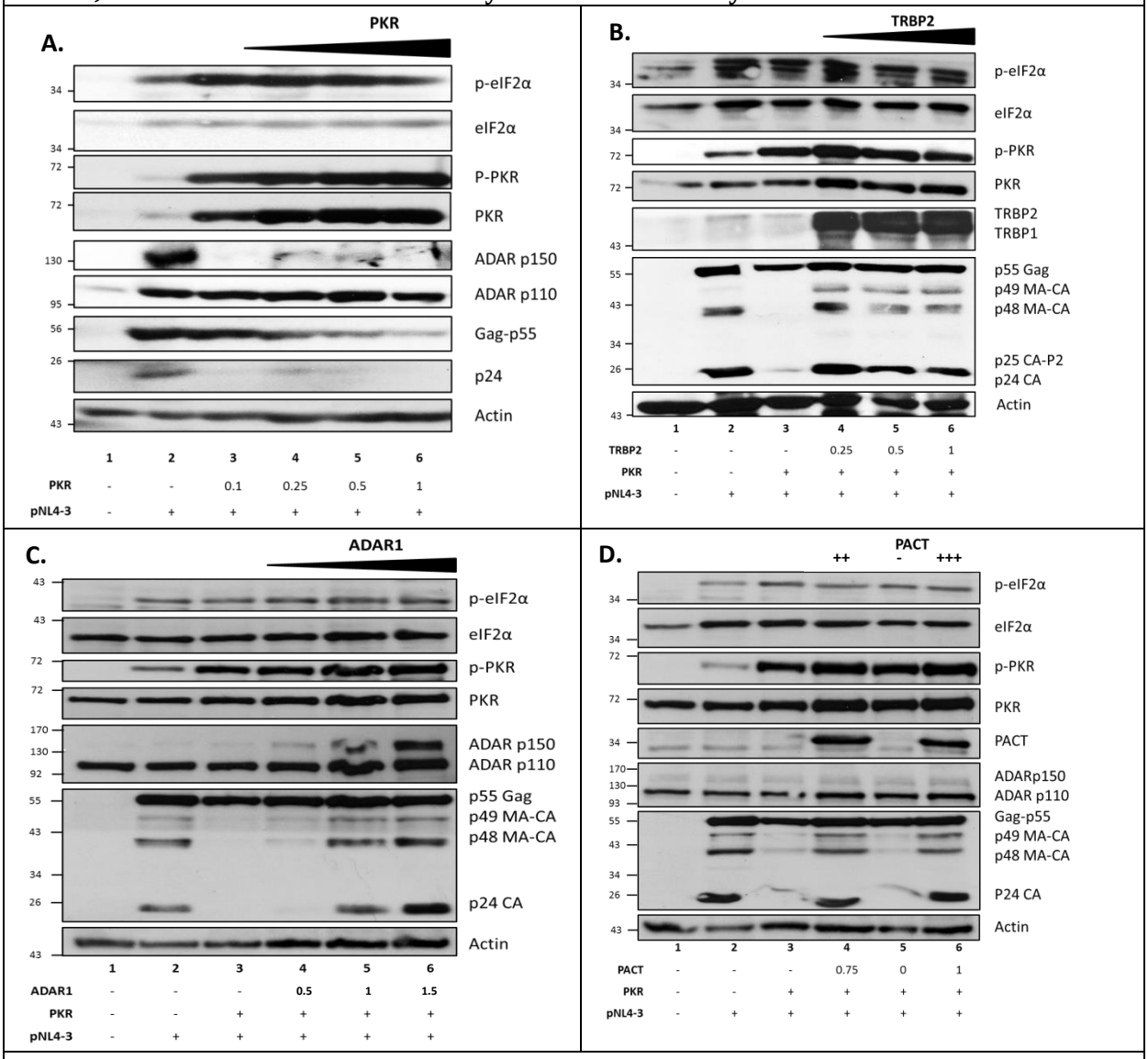
Transfection were normalised at 1.1µg of DNA per well by compensating with corresponding empty plasmids (pCMV Myc empty, pCDNA 3.1 empty, pCMV Flag empty).

This experiment shows the inhibitory effect of PKR on the expression of the HIV LTR in HEK293T cells and the inhibitory effect of ADAR1, TRBP and PACT on PKR antiviral activity. We then decided to test the effect of PKR on the production of viral proteins.

Inhibition of HIV viral protein production by PKR and interaction with ADAR1, TRBP and PACT in HEK293T:

In this set of experiments we investigated the antiviral effect of PKR on the expression of HIV viral proteins. Viral protein expression can be rescued from PKR inhibition if the ADAR1, TRBP or PACT proteins are also overexpressed. This set of experiments was designed to determine the antiviral function of PKR on the expression of viral proteins from an HIV molecular clone and the inhibitory effect of ADAR1, TRBP and PACT and PKR activity. The HEK293T cell line was transfected with HIV-1 molecular clone and plasmids expressing PKR, ADAR1, TRBP and PACT.

Figure 8. Inhibitory effect of PKR on HIV-1 replication in HEK 293T, counteracted by TRBP, PACT and ADAR1 assessed by Western blot assay.



The HEK 293T cell line was plated on 6 well culture plates (Corning) and transfected with **A.** 1µg of a molecular clone of HIV (pNL4-3) and increasing doses (0.1µg, 0.25µg, 0.5µg, 1µg) of a plasmid expressing PKR (pcDNA 1 PKR) **B.** 1µg of a molecular clone of HIV (pNL4-3), 0.25µg of pcDNA 1 PKR and incremental doses (0.25µg, 0.5µg, 1µg) of a plasmid expressing TRBP2 (pCMV Myc TRBP2) **C.** 1µg of a molecular clone of HIV (pNL4-3), 0.25µg of pcDNA 1 PKR and incremental doses (0.5µg, 1µg, 1.5µg) of a plasmid expressing ADAR1 (pDNA 3.1 ADAR) **D.** 1µg of a molecular clone of HIV (pNL4-3), 0.25µg of pcDNA 1 PKR and different doses (0.75µg, 0µg, 1µg) of a plasmid expressing PACT (pCMV Flag PACT). In Panels A, B, C and D cellular lysates (50 to 90 µg) were loaded into a 10% SDS PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and Enhanced Chemiluminescence (ECL) solution was used for revealing.

In the experiments presented in **Figure 8,** we characterised the levels of activation and expression of the different proteins of the PKR pathway in the presence of HIV in the cell. Cells were transfected with 1µg of a molecular clone of HIV (pNL4-3). Cells were lysed and protein quantities were normalised by Bradford assay. In **A,** incremental doses (0.1µg, 0.25µg, 0.5µg, 1µg) of a plasmid expressing PKR (pcDNA 1 PKR) were then transfected. Overexpressing PKR inhibits the expression of the viral proteins p24 and p55 Gag. In **B, C,** and **D** cells were transfected with 1µg of pNL4-3, 0.25µg of pcDNA 1 PKR and incremental doses of pCMV Myc TRBP, pcDNA 3.1 ADAR1 and pCMV Flag PACT. The viral protein production is inhibited when PKR is transfected and then restored when TRBP2, ADAR1 or PACT are overexpressed. The apparent activation of ADARp150 in panel **A** can be explained by the fact that ADAR p150 and ADAR p110 were exposed separately as opposed to **C** and **D** where ADAR p110 and ADAR p150 were exposed together. ADAR p150 gives a weaker signal compared to ADAR p110 which explains why ADAR p150 seems to be induced when pNL4-3 alone is transfected in **A** but not in **C** and **D.**

This experiment shows the inhibitory effect of PKR on the expression of HIV proteins in HEK293T cells and the inhibitory effect of ADAR1, TRBP and PACT on PKR antiviral activity. PKR has an antiviral function which is counteracted by cellular factors ADAR1, TRBP and PACT. These mechanisms allow active viral replication in productively infected cells; we then decided to investigate the expression and activation of the PKR pathway in latently infected cells.

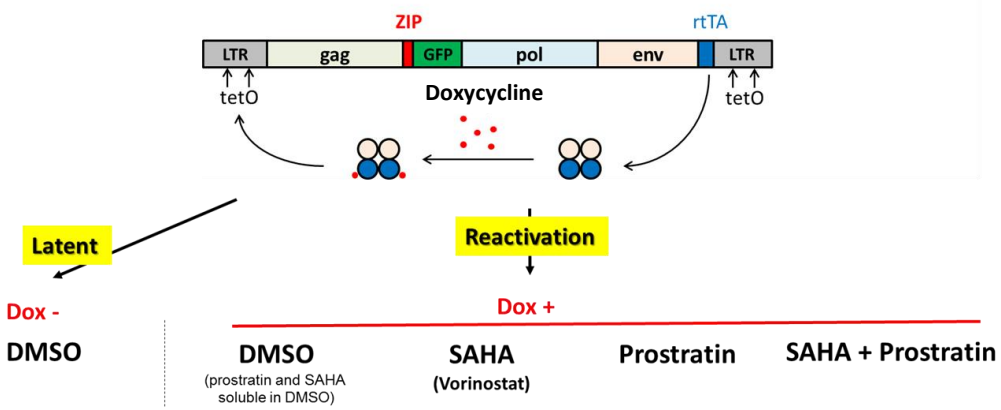
Project 2 – Using an HIV-1 model to study post transcriptional control of viral replication by the PKR pathway

In this project we investigated the activation and transcription levels of the PKR pathway in two cell line latency models. Both models were based on a T lymphocyte cell line (CEM T4) and a Monocytes-derived Macrophages (MDM) cell line (THP-1) with an integrated modified HIV-1 provirus. This model was used to determine the role of PKR in the establishment and maintenance of latency.

Presentation of the HIV-1 latency model:

The model used was based on a model developed by (Das et al., 2004) and consists of an HIV-1 provirus integrated to the cell genome. Tat and its TAR RNA binding sequence are mutated and inactivated. Tat and TAR are replaced by the reverse Tc-controlled Transcriptional Activator (rtTA), the transcriptional activator protein and its target site, the tet operator (tetO). To monitor the reactivation of each cell, a GFP reporter is fused to the Gag protein with a Zip sequence. The Reverse Transcriptase is mutated and no viral particle is produced. The HIV-1 latent model provirus genome layout is presented in **Figure 9.**

Figure 9. HIV-1 latent model provirus genome layout



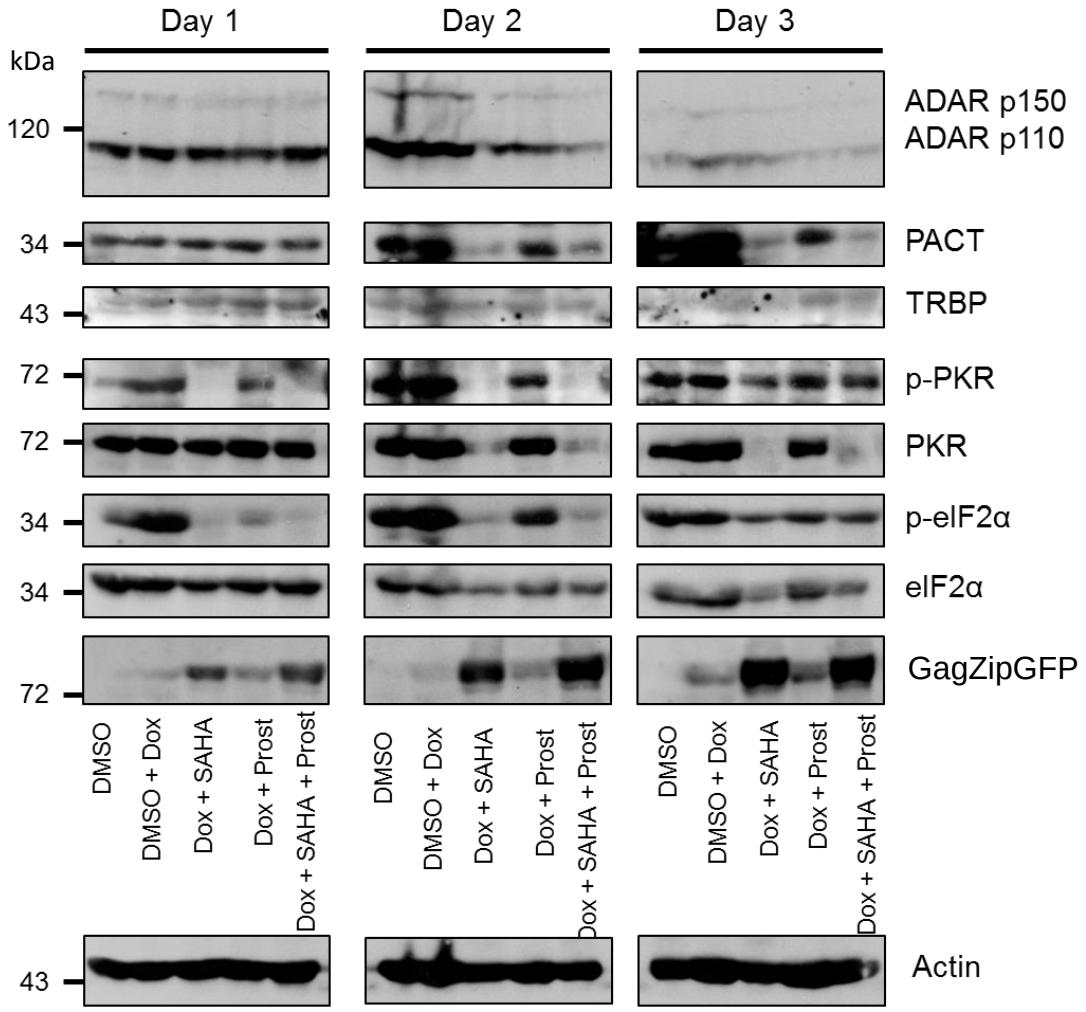
Genome layout of the latent HIV-1 model and reactivation regimens. The model was engineered by Dr. Alan Cochrane and based on a model developed by Das et al. (2004). The integrated provirus is under the control of a Tetracycline-Controlled Transcriptional Activation (tet-On/tet-Off) and is dependent on Doxycycline, a derivative of tetracycline (Tc). For the reactivation, two different LRAs were selected SAHA (suberanilohydroxamic acid or vorinostat) and Prostratin. The protein Gag is fused to a GFP reporter with a Zip sequence, which is used to monitor reactivation.

In this model the modified lentivirus has been integrated into the chromosome of the lymphocyte CEM T4 and the monocyte/macrophage THP-1 cell lines. The THP-1 cell line was differentiated into macrophages using PMA. Optimal LRA quantities required for reactivation were determined by Dr. Elodie Rance in our laboratory (Rance et al., in preparation).

Reactivation of the latent model at days 1 to 3 and effect on the PKR pathway in CEM T4 GagZipGFP:

After the reactivation regimens were determined by Dr. Elodie Rance in our laboratory, we tested the activation and expression levels of the PKR pathway at days 1, 2 and 3 in the CEM T4 GagZipGFP cell line.

Figure 10. Activation and expression of the PKR pathway in the CEM T4 GagZipGFP cell line after reactivation at days 1, 2 and 3 assessed by Western blot analysis.



For each condition, cells were cultured in T75 culture flasks (VWR). Cells were then treated with DMSO, DMSO +Doxycyclin, Doxycyclin+SAHA, Doxycyclin+Prostratin and Doxycyclin+SAHA+Prostratin. Cells were lysed at day one two and three, protein quantities were normalised by Bradford assay. Cellular lysates (50 to 90 µg) were loaded into a 10% SDS PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and revealed after adding an Enhanced Chemiluminescence (ECL) solution.

In the experiment presented in **Figure 10,** the CEM T4 GagZipGFP cell line was reactivated using the regimens listed above (see the method section for concentrations). Strong reactivation was observed when cells were treated with SAHA. This strong reactivation coincided with a strong inhibition of PKR activation (day 1-2) and expression (day 2-3). This was

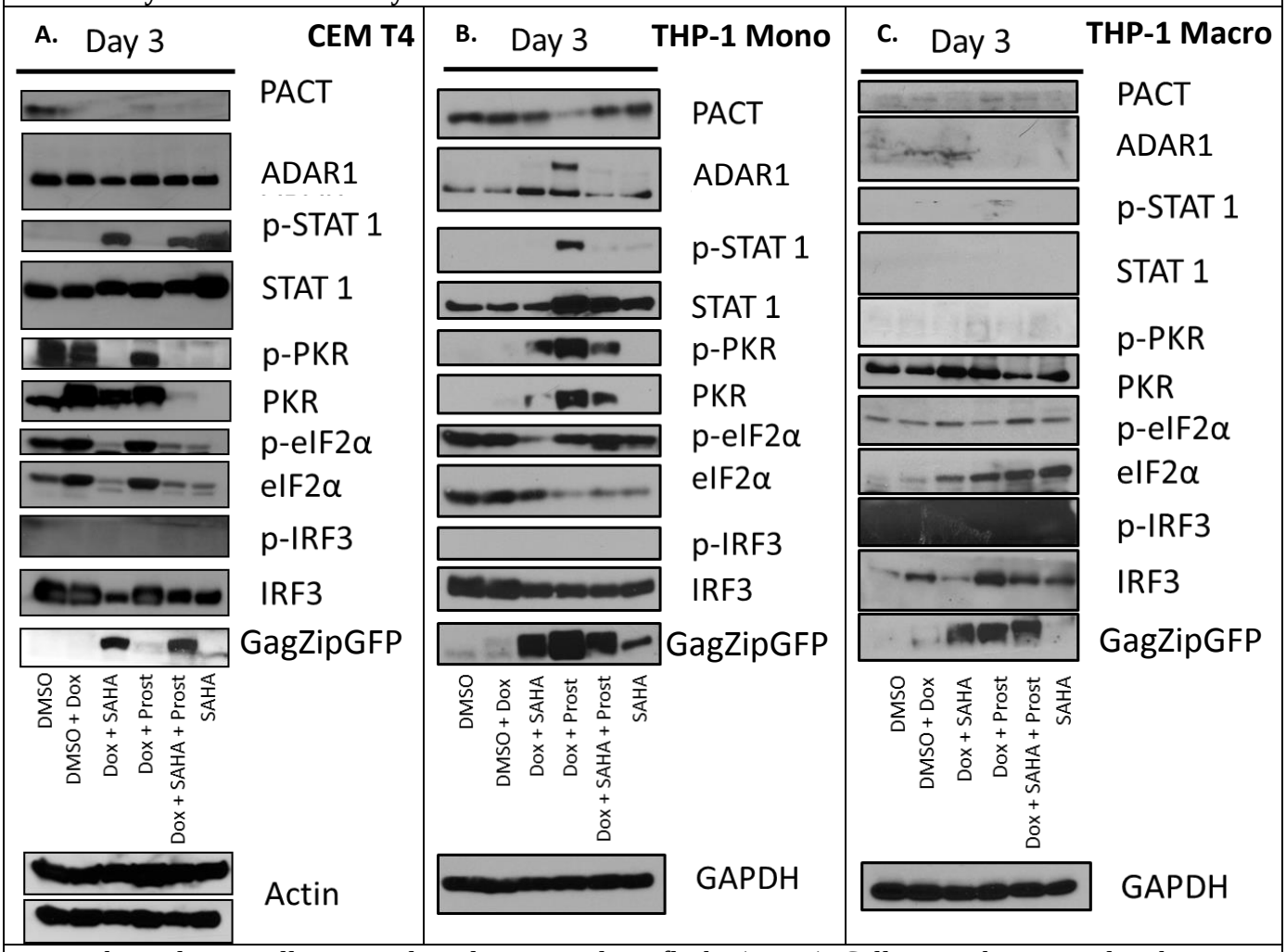
correlated with an inhibition of eIF-2α activation (day 1-2) and expression (day 2-3). The expression of the PKR activator PACT was also inhibited (day 2-3).

This strong inhibition of PKR expression and activation correlating with a strong expression of the HIV GagZipGFP protein construct in cells treated with SAHA (Figure 10, lanes with SAHA) suggested a link between SAHA mechanism of action and reactivation from latency in CEM T4 GagZipGFP cells. To confirm these findings, we tested the activation and expression of the PKR pathway in the CEM T4 and THP-1 cell lines.

Reactivation of the latent model at day 3 and effect on the PKR pathway in CEM T4 GagZipGFP and THP-1 GagZipGFP:

To assess the effect of reactivation in the lymphocytic and monocytic/macrophage lineages, we tested the activation and expression levels of the PKR pathway at day 3 both CEM T4 GagZipGFP and THP-1 GagZipGFP.

Figure 11. Activation and expression of the PKR pathway in the CEM T4 GagZipGFP, the THP-1 GagZipGFP Monocyte and THP-1 GagZipGFP Macrophage cell lines after reactivation at day 3 assessed by Western blot assay.



For each condition, cells were cultured in T75 culture flasks (VWR). Cells were then treated with DMSO, DMSO +Doxycyclin, Doxycyclin+SAHA, Doxycyclin+Prostratin and Doxycyclin+SAHA+Prostratin and SAHA alone as indicated. Cells were lysed at day three, protein quantities were normalised by Bradford assay. Panel A corresponds to the CEM T4 cell line, Panel B to the THP-1 monocytes and panel C to the THP-1 macrophages. Cellular lysates (50 to 90 µg) were loaded into a 10% SDS PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and revealed after adding an Enhanced Chemiluminescence (ECL) solution.

The same experiment as in figure 9 was repeated with the CEM T4 GagZipGFP, the THP-1 GagZipGFP monocyte and THP-1 GagZipGFP macrophage cell lines on day 3. A condition where cells were treated with SAHA alone was added to assess the effect of SAHA on the PKR pathway. In the CEM T4 cell line the same inhibitory effect was observed on PKR expression and activation, eIF-2α expression and activation and PACT expression. The condition with

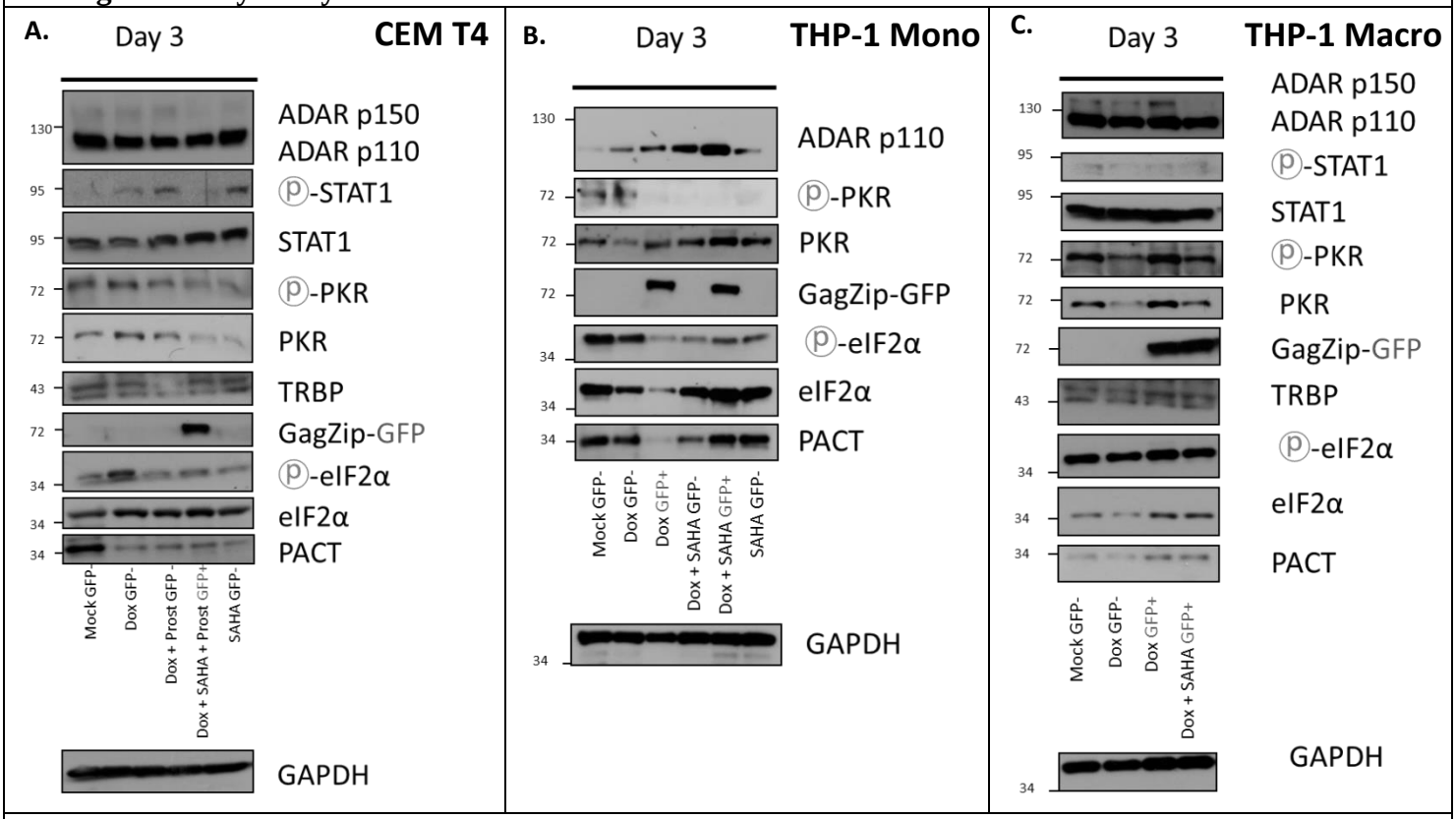
SAHA alone had similar effects but did not induce strong reactivation as the model is dependent on doxycycline for reactivation. Interestingly SAHA stimulated the expression and activation of STAT1, a protein involved in IFN response which should activate PKR expression (PKR being an ISG). The increase of STAT1 expression and the concomitant PKR inhibition in lymphocytes is currently not understood, but suggests a differential activity at the transcriptional level.

These effects on the PKR pathway were not observed in the THP-1 macrophage and monocyte cell lines. SAHA does not seem to reactivate cells more than prostratin and PKR seems to be activated in the presence of SAHA. In the THP-1 macrophage cell line STAT1, p-STAT1 and ADAR1 could not be detected. To refine the results obtained in this experiment, we then sorted cells using GFP as the reactivation marker to separate cells into treated-reactivated cells and treated-non-reactivated cells.

Reactivation of the latent model at day 3, FACS sorting of reactivated and non-reactivated cells and effect on the PKR pathway in CEM T4 GagZipGFP and THP-1 GagZipGFP:

As outlined in the results presented in the appendixes, when cells are treated with each reactivation regiment, not all cells are reactivated. In this experiment we wanted to refine our understanding of the reactivation pattern and the effects observed in the previous experiment on the PKR pathway. Using the GFP as the reactivation marker, we separated cells into reactivated and non-reactivated subgroups for each reactivating condition. We then assessed the effect of reactivation on both CEM T4 GagZipGFP and THP-1 GagZipGFP we tested the activation and expression levels of the PKR pathway at days 3.

Figure 12. Activation and expression of the PKR pathway in the CEM T4 GagZipGFP, the THP-1 GagZipGFP Monocyte and THP-1 GagZipGFP Macrophage cell lines after reactivation at day 3, FACS sorting and analysed by Western blot.



For each condition, cells were cultured in T75 culture flasks (VWR). Cells were then treated with DMSO, DMSO+Doxycyclin, Doxycyclin+SAHA, Doxycyclin+Prostratin and Doxycyclin+SAHA+Prostratin. Cells were sorted by FACS using GFP as the selection marker. Cells were lysed at three, protein quantities were normalised by Bradford assay. Panel A. corresponds to the CEM T4 cell line, Panel B. to the THP-1 monocytes and panel C. to the THP-1 macrophages. Cellular lysates (50 to 90 µg) were loaded into a 10% SDS PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and revealed after adding an Enhanced Chemiluminescence (ECL) solution.

In this experiment, we wanted to assess the effect of SAHA and to compare the activation and expression profile of the PKR pathway in non-treated, treated-reactivated and treated-non-reactivated cells at day 3. Cells were sorted by FACS using GFP as the selection marker for reactivation. Some cell populations were too small to produce enough protein to perform a western blot and were then ruled out.

After testing the expression and activation after sorting, the data was then summarised in a table outlining the effect of SAHA treatments for added clarity.

Overview of the effects of SAHA treatments on CEM T4 GagZipGFP and THP-1 GagZipGFP:

Figure 13. Overview of the effect of SAHA on the PKR pathway in the CEM T4 GagZipGFP, the THP-1 GagZipGFP Monocyte and THP-1 GagZipGFP Macrophage cell lines after FACS sorting.

CEM T4 Lymphocytes			THP-1 Monocyte		THP-1 Macrophages	
SAHA treatments	GFP+	GFP-	**GFP+ Cells**		**GFP+ Cells**	
ⓟ-PKR	↓	↓	ⓟ-PKR	↓	ⓟ-PKR	→
PKR	↓	↓	PKR	→	PKR	→
PACT	↓	↓	PACT	→	PACT	→
ⓟ-eIF2α	↓	↓	ⓟ-eIF2α	↓	ⓟ-eIF2α	→
eIF2α	→	→	eIF2α	→	eIF2α	↑
p24	↑	↓	p24	↑	p24	↑
ⓟ-STAT1	↓	↑				

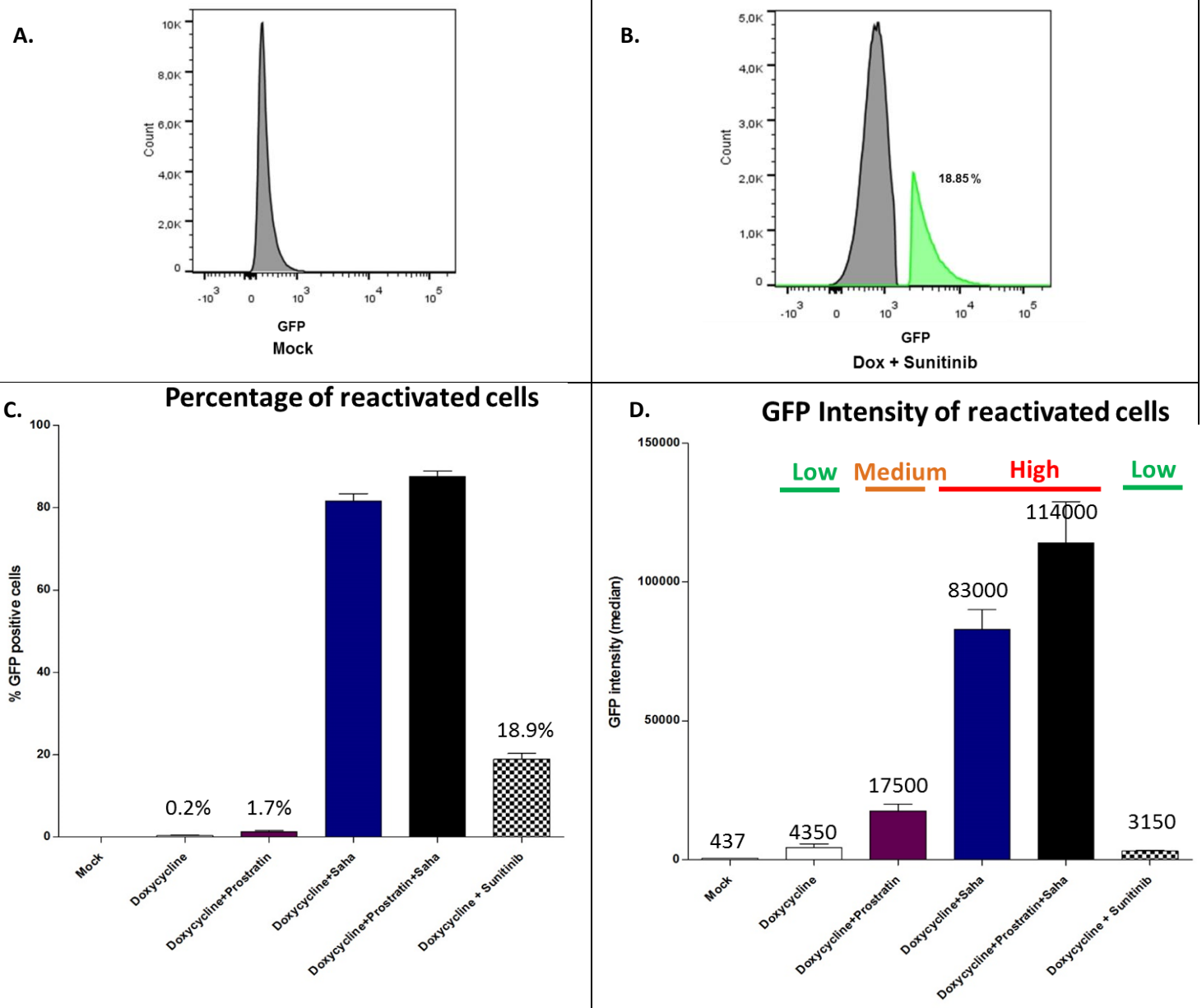
This figure summarises the effect of SAHA on each protein of the PKR pathway listed assed in the **figure 11**. The red arrows signify a decrease in the band intensity for the protein tested by Western blot, blue arrows signify a stable intensity and green arrows signify an increase in intensity.

Figure 13 summarises the findings of **Figure 12** in the cells treated with SAHA (reactivated GFP^+ and non-reactivated GFP^-). In the CEM T4 cell line, the PKR pathway seems to be largely inhibited in both GFP^+ cells and GFP^- cells. The main difference observed is the activation of STAT1 (increased levels of p-STAT1) in the treated non-reactivated cells. For the THP-1 monocyte and macrophage cell line the PKR pathway is minimally affected by SAHA treatments whereas it is strongly observed in the lymphocytic cell line. To investigate the role of the PKR inhibition observed in cells treated with SAHA and evaluate if PKR inhibition contributes to latency reversal, we tested PKR inhibitors on the latent model.

Using a PKR inhibitor to reactivate the CEM T4 GagZipGFP cell line:

The extent of the reactivation was measured after treatment of the CEM T4 GagZipGFP cell line with the PKR inhibitor sunitinib. Cells were analysed by FACS and Western blot to explore HIV reactivation and PKR phosphorylation to determine if Sunitinib could be used as a LRA.

Figure 14. Reactivation pattern of CEM T4 GagZipGFP cells after treatment with Doxycyclin and Sunitinib



Testing the PKR inhibitor Sunitinib as a potential LRA **A.** Mock reactivation (DMSO) for the CEM T4 GagZipGFP cell line FACS cell count and **B.** Doxycyclin + Sunitinib treatment of the CEM T4 GagZipGFP cell count with percentage of reactivated cells. **C.** Percentage of reactivated cells (GFP positive) of the CEM T4 cell line after treatments **D.** Intensity of GFP expression for each treatment (Median Fluorescence Intensity or MFI) **E.** Western blot assessing the viral protein expression (GagZipGFP) PKR expression and activation and eIF-2α expression and activation in Sunitinib treated cells (0.3µM, 0.6µM and 1µM)

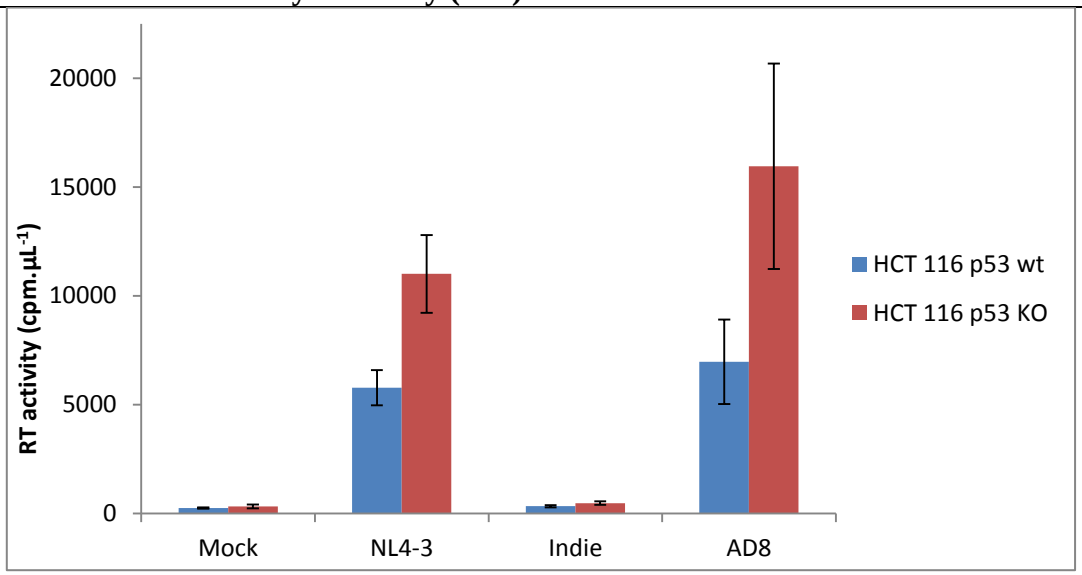
In the experiment presented in **Figure 14,** we tested the effect of Sunitinib an inhibitor of PKR on the reactivation of the CEM T4 cell line. We hypothesised that SAHA will produce a strong reactivation of the provirus in this cell line partly through the inhibition of the PKR pathway. Our results show an 18.9% reactivation of the cells treated with Sunitinib and Doxycycline. However, the intensity of the GFP expression in the reactivated cells treated with Sunitinib and Doxycycline was lower than when cells were treated with SAHA suggesting that PKR activation may not be the sole mechanism in HIV reactivation by SAHA but likely contributes to it.

Treating the CEM T4 cell line with the PKR inhibitor Sunitinib has a reactivation potential, which suggests that PKR plays a role in the establishment of latency in this cell line and that Sunitinib could be used as a Latency Reversing Agent.

Project 3 – The role of the p53 pathway during HIV infection

In the context of a collaboration with Dr. David Reisman from the Department of Biological sciences, University of South Carolina we explored if p53 affects HIV production with the aim to analyse the possible connection between p53 and the PKR pathway (Yoon et al., 2015). Because most cell lines commonly used to replicate HIV are mutated or inactivated for p53, we chose a pair of the same cells with only one Knocked down for p53. Dr. Reisman provided us with two cell lines: HCT 116 p53wt and HCT 116 p53 KO. The HCT 116 p53wt expresses a wild type version of p53 and HCT 116 p53 KO does not express p53. We transfected these two cell lines with different molecular clones of HIV and observed the effect on viral production by RT assay. The increased expression of HIV expression in HCT 116 p53KO compared to HCT 116 p53wt when transfected with HIV-1 molecular clones is presented in **Figure 15.**

Figure 15. Increased expression of HIV expression in HCT 116 p53KO compared to HCT 116 p53wt when transfected with the molecular clones of HIV-1 pNL4-3, Indie and AD8 and measured by RT assay (n=6).



The two cell lines HCT 116 p53 wt and HCT 116 p53 KO were both plated at 70% confluence on 12 well culture plates (Corning). Cells were transfected with HIV molecular clones pNL4-3, pIndie and pAD8 using transit-LT1 (Mirus) transfecting reagent. pBluescript was used as control. After 48h 5µL of supernatant were collected and used to perform a RT assay. RT is expressed in cpm.µL^{-1} ISEM (n=6).

In this experiment, we investigated the effect of p53 on viral production in two stable cell lines HCT 116, one expressing wild type p53 and one p53 Knock-Out. For each condition, cells were transfected with 1µg of viral plasmid or pBluescript using the transIT-LT1 (Mirus) transfecting reagent. Supernatants were tested by RT assay after 48h. We observed a higher viral replication in supernatant from the HCT 116 p53 KO cell line compared to HCT p53 wt.

This experiment shows that p53 could play a role in the inhibition of HIV production in various cells as suggested previously (Yoon et al., 2015). The interplay between the p53 and PKR pathways could be a mechanism controlling latency in certain cell types and could help to understand the reactivation pattern observe in project 2.

DISCUSSION

PKR is an ISG, with well documented antiviral activity during HIV-1 and other viral infections. PKR primary antiviral activity is carried out by its kinase activity and the phosphorylation of the eukaryotic initiation factor eIF-2α. During HIV infection, PKR is transiently activated and then inhibited by direct interactions with ADAR1, TRBP and PACT. Interactions between HIV and PKR could be responsible for the establishment and maintenance of latency. Latency is one of the main obstacles to the development of a curative HIV therapy. Finally, interplay between PKR and the p53 pathway could also play a role in this process.

1- Inhibition of viral replication by the PKR pathway and inhibition of PKR by ADAR1, TRBP and PACT

Our laboratory has previously shown that PKR is transiently activated and then inhibited during active HIV replication. This set of experiments was designed to corroborate these findings and to serve as an introduction to the project. The first experiment consisted in testing the effect of PKR overexpression on the expression of a luciferase reporter under the control of the HIV promoter (LTR). As predicted, PKR overexpression inhibited the expression of the luciferase reporter which is in line with the antiviral activity of PKR documented in the literature. The next experiments introduced PKR inhibitors ADAR1, TRBP2 and PKR activator PACT that has previously been shown to become a PKR inhibitor during active HIV replication by our lab. The overexpression of ADAR1, TRBP2 and PACT inhibits PKR and allows a restoration of the luciferase reporter gene expression. This PKR inhibition by ADAR1 and PACT was dose dependent whereas TRBP inhibition of PKR reached a threshold at which its activity was reversed.

In a second set of experiments we confirmed previous findings by Clerzius et al. (2013); PKR overexpression inhibits the expression of an HIV molecular clone pNL4-3 in HEK293T. We monitored by Western blot the effect of PKR overexpression on the PKR pathway. We then tested the effect of overexpression of ADAR1, TRBP2 and PACT on pNL4-3 expression and PKR induced inhibition. As demonstrated Clerzius et al. (2013) by PKR inhibits the expression of pNL4-3 viral proteins in a dose dependent manner and this inhibition correlates with an increase in eIF-2α phosphorylation and expression. The subsequent overexpression of ADAR1, TRBP2 and PACT has an inhibitory effect on PKR and eIF-2α phosphorylation and transcription correlated to a dose-dependent increase in viral protein expression.

These results correlate with previous studies undertaken in our laboratory (Clerzius et al., 2009, Clerzius et al., 2013, Daher et al., 2001). They illustrate the antiviral effect of PKR as well as the inhibitory effect of ADAR1, TRBP2 and PACT on PKR antiviral activity (reviewed in (Clerzius et al., 2011, Burugu et al., 2014)).

2- Using an HIV-1 model to study post transcriptional control of viral replication by the PKR pathway

In the framework of the pan-Canadian collaboration on HIV research CanCure, our laboratory received an HIV latency model engineered by Dr. Alan Cochrane from the University of Toronto in two cell lines. This model is based on a previous model engineered by Das et al. (2004). In the model engineered by Dr. Cochrane's lab, a modified non-productive HIV-1 is integrated in a monocytic/macrophage cell line (THP-1) and a CD4+ lymphocytic cell line (CEM T4). The protein expression is dependent on a tet/on tet/off system induced by doxycycline and contains a GFP reporter gene to monitor reactivation. This reporter is fused to the Gag protein by a Zip sequence and produces a GagZipGFP construct when reactivated. In addition to requiring the introduction of doxycycline for reactivation, this latency model requires the addition of LRAs for a potent reactivation. This conditional reactivation represents a clear advantage to study latency. Indeed, the conditions can be effectively controlled with very little leakage into reactivation. Although this reactivation pattern does not completely reproduce what may be happening *in vivo* this model provides a useful and powerful tool to study a complex mechanism such as latency.

Reactivation regimens were tested and optimised by Dr. Elodie Rance in our laboratory (Rance et al., in preparation). These reactivation regimens were originally designed to study the miRNA profiles in latently infected cells and the effects of reactivation.

The first set of experiments was designed to investigate the effect of latency and reactivation on the PKR pathway. The CEM T4 cell line was selected first because lymphocytes represent the main HIV reservoir in infected individuals. Cells were treated with the reactivation regimens established by Dr. Elodie Rance and cells were lysed at days one, two and three and the expression and activation of the PKR pathway was assessed by Western blot. The main effect

observed in this experiment was a strong expression of the reactivation reporter GagZipGFP when cells were treated with the HDAC inhibitor SAHA (vorinostat). This reactivation correlated with an inhibition of PKR activation and transcription most noticeably at day 3. This inhibitory effect was also observed on the transcription of the PKR activator PACT and on the activation of eIF-2α. These observations were in line with our initial hypothesis that the PKR pathway is engaged in the establishment and maintenance of latency.

We decided to widen the study to the macrophage and monocytic cell models. To further characterise the role of PKR and define the effect of SAHA, a condition was added where cells were treated with SAHA alone, which should not trigger reactivation as doxycycline is not present. PKR is an ISG and to assess the IFN pathway activation STAT1 expression and activation was also monitored. As the strongest reactivation and most of the effects on the PKR pathway were observed at day 3, we retained this time point for our analysis. The effects observed in the previous experiment were confirmed in the CEM T4 cell line. The additional condition with SAHA alone showed the same effect on the PKR pathway with a much lower reactivation: the GagZipGFP reporter expression was low but still observable, SAHA being a very potent reactivator in this cell line. This observation that SAHA is more likely to directly affect the PKR pathway and the effect observed were not mediated by the expression of viral factors. Interestingly, STAT1 activation was been induced by SAHA which should in turn activate the expression of PKR. Although further investigation is required, this suggests that SAHA affects the PKR pathway downstream of the IFN pathway. SAHA did not have the same effect in the THP-1 macrophage and monocytic cell lines. In addition, many of the proteins tested such as STAT1, p-STAT1 and ADAR1 could not be detected. This might be due to the difficulty in growing sufficient quantities of cells combined with high cell death induced by the reactivation regimens and the differentiation protocol in macrophages. We decided to focus our study on the CEM T4 cell line as monocytes and macrophages are a less well defined reservoir and there is

still debate around the clinical relevance of this reservoir in the resurgence of viremia after cessation of cART (Abbas et al., 2015).

One of the important aspects of reactivation is that not all cells are reactivated from latency when treated with the reactivation regimens. To further refine our understanding of the mechanisms involved during reactivation, we decided to sort cells to compare treated-reactivated and treated-non-reactivated cell populations. The same experiment was repeated with the introduction of cell sorting using the GFP reporter as the selection marker for reactivation. In the CEM T4 cell line, we observed the same effect of SAHA as in the previous experiments; a high level of reactivation coupled with a strong inhibition of the PKR pathway. In the previous experiment, we also observed an activation of STAT1 in cells treated with SAHA. The cell sorting allowed refining this observation; STAT1 is only activated in the stimulated-non-reactivated population. This indicates that STAT1 is involved in the latency maintenance as it is activated in the non-reactivated populations and is inactivated in reactivated populations. In the THP1 macrophage and monocyte cell lines, the involvement of the PKR pathway seems to be far less engaged. As previously observed, there is no discernable pattern even after sorting cells. The THP-1 cell line was not retained for further investigation.

The alteration of the PKR pathway by the treatment with SAHA in the CEM T4 cell line correlated with a strong reactivation pattern led us to hypothesise that the PKR pathway could be targeted to induce reactivation from latency. We selected the PKR inhibitor Sunitinib and decided to test its effect on reactivation. Cells were treated with Sunitinib and doxycycline followed by FACS sorting using the GFP reporter as the reactivation marker. The Sunitinib treatment induced a significant population to reactivate (18.9% GFP positive cells). However, the reactivated population had lower level of expression of GFP, suggesting a lower expression of viral proteins. Low levels of reactivation are also observed in the reactivation condition when doxycycline alone

is used with a much smaller population of cells reactivated (0.2% of GFP positive cells). This experiment shows that targeting PKR could represent a valid target for the discovery of an entire novel class of LRAs targeting post transcriptional control of HIV replication. The low intensity of GFP indicates that although reactivated the integrated provirus is expressed at lower levels than when treated with other LRAs tested such as SAHA and Prostratin. Using LRAs in combination with PKR inhibitors such as Sunitinib could be considered for inducing a more potent reactivation. One of the limitations of this study is that it was done using a cell line model and may be only partly representative of what is happening *in vivo*. However, it is a strong indication that post transcriptional control and more specifically control by the PKR pathway, could be involved in the establishment and maintenance of latency in the Lymphocytic reservoir.

The next step in this investigation would be to test this hypothesis in a different latency model and test different PKR inhibitors. Further testing of PKR inhibitors in latently infected cells from patients would also be required to prove the efficacy of PKR inhibitors in reactivating HIV latent reservoirs.

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Interplay between the PKR and p53 pathways during HIV infection

Our lab was contacted by Dr. David Reisman from the University of South Carolina to collaborate on investigating the role of p53 in the replication of HIV. In the light of a recent publication by Yoon et al. (2015), the PKR and p53 pathways seem to interact and inhibit the replication of HIV. Dr. Reisman's lab provided us with two cell lines based on the HCT 116 cell line. Most cell lines used to replicate HIV have a mutated version of p53 as p53 has been shown to inhibit HIV replication. The first cell line is an HCT 116 cell line with a wild type version of p53 (p53 wt) and the second is an HCT 116 cell line a Knocked Out version of p53 (p53 KO).

To observe the effect of p53 on the replication of HIV in the HCT 116 cell line, cells were transfected with different molecular clones of HIV. Cells were plated at 70% density and incubated for 48h after transfection. The supernatant were then collected and the RT activity was measured. Our results show that the HCT 116 p53 KO cell line produced more virus than the HCT 116 p53 wt cell line, suggesting a role of p53 in restricting HIV replication.

This preliminary data corroborate the findings by (Yoon et al., 2015) in two new cell lines. This experiment provides us with two cell lines capable of replicating an HIV molecular clone and can be used to assess the role of p53 during HIV replication. This experiment was also set up as a stepping stone for investigating the potential of p53 as another potential target to reverse latency in our latent model.

CONCLUSION

PKR is an ISG with well documented antiviral function against multiple viruses including HIV-1. The PKR pathway is engaged in the post transcriptional control of HIV replication and may play a role in the establishment of latently infected cellular reservoirs.

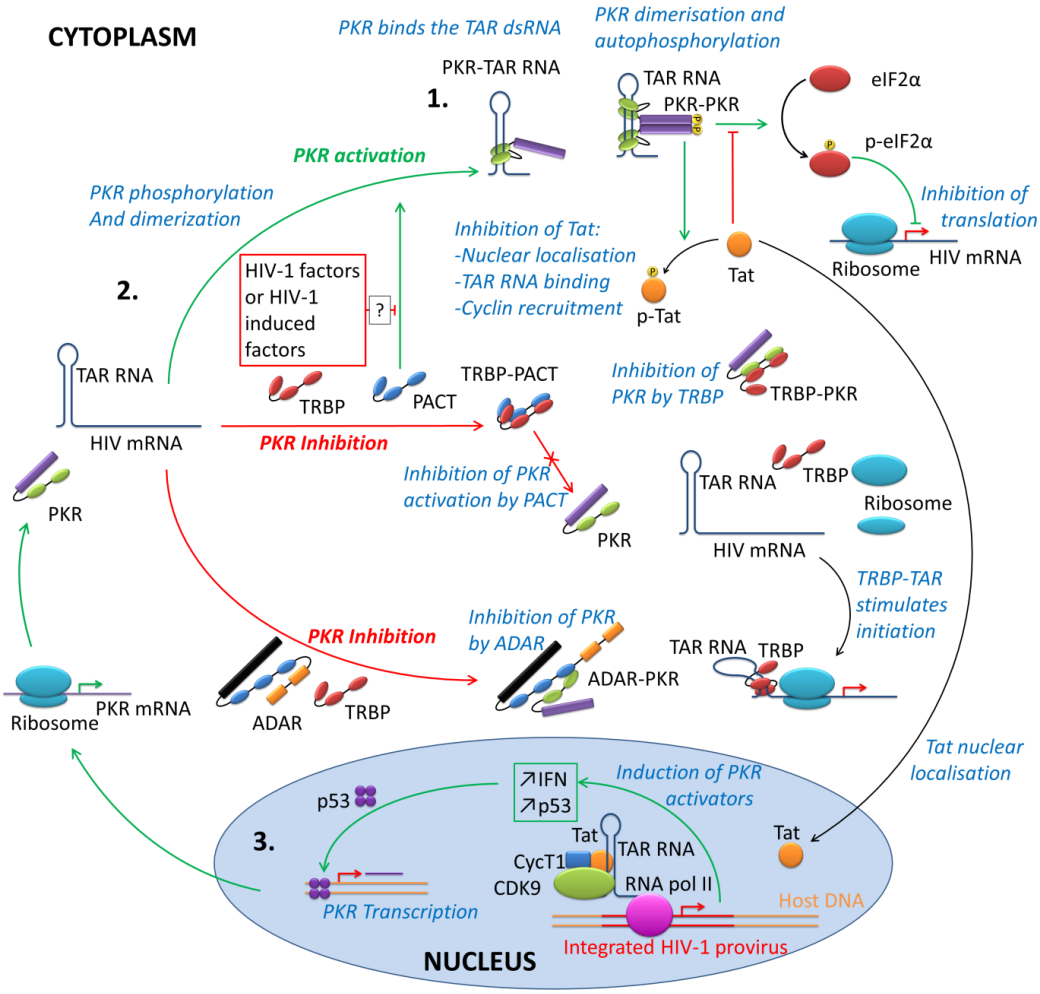
Using a latency model we demonstrated that the PKR pathway is affected when using the HDAC inhibitor SAHA for reactivation. We observed a direct correlation between the expression of the provirus and the inhibition of the PKR pathway in the lymphocytic cell line CEM T4.

The inhibition of the PKR pathway was further investigated and the activation of STAT 1, a member of the IFN pathway was assessed. STAT1 was activated in the cells treated with SAHA. Cell sorting revealed that STAT1 was activated in cells that were stimulated-not-reactivated cells and inactive in cells that were treated-reactivated. This indicated that the IFN signalling pathway could be a critical component in the reactivation process.

To further investigate the involvement of the PKR/IFN pathway cells were treated with a PKR inhibitor Sunitinib to test the reactivation potential of targeting PKR. This experiment revealed that almost 20% of CEM T4 cells could be reactivated using this compound. This study lays the foundation for future investigations of targeting the post transcriptional control of HIV to reactivate latently infected cells.

In parallel to this study, we tested the impact of p53 on HIV replication in the HCT 116 cell line. This experiment revealed that p53 inactivation allows HIV to better replicate when transfected with an HIV molecular clone. This is in line with recent publications and is a potential target for latency reactivation. We summarised our findings together with previous published results in a schematic integrating the p53 and PKR pathways during HIV replication (**Figure 16**).

Figure 16. The p53 and PKR restriction of HIV-1 and viral countermeasures



1. **PKR activation and counteraction by Tat during HIV-1 infection**
   - PKR binds the double stranded HIV TAR RNA through its two dsRBDs. PKR is then activated by homodimerisation and autophosphorylation.
   - Activated PKR phosphorylates eIF2α which inhibits translation of viral and cellular mRNAs.
   - HIV protein Tat inhibits PKR kinase activity by acting as a competitive inhibitor. Phosphorylation of Tat on multiple sites inhibits its transactivation activity by inhibiting its nuclear localisation, its binding to the TAR RNA and its recruitment of CycT1 and CDK9.
2. **Inhibition of PKR activation by cellular factors during HIV replication**
   - PKR is inhibited by direct interaction with TRBP. TRBP also inhibits PKR activation by sequestering the PKR activator PACT.
   - TRBP promotes HIV mRNA translation by direct binding to the TAR RNA. This relaxes the structure of TAR and promotes the recruitment of initiation factors and binding of the ribosome.
   - PKR is inhibited by direct interaction with ADAR1.
   - PACT is converted into a PKR inhibitor during HIV active replication by unknown HIV factors or HIV induced cellular factors.
3. **HIV-1 restriction by PKR and p53 and Tat counteraction**
   - Tat is expressed during HIV replication and translocates to the nucleus. Tat binds TAR when HIV mRNA is transcribed and recruits CycT1 and CDK9 (pTEFb). pTEFb hyperphosphorylates the RNA pol II. Recruitment of Tat and pTEFb to TAR releases a translational block and stimulates the expression of the HIV genome.
   - Tat activity is also inhibited by PKR phosphorylation. PKR phosphorylates Tat at multiple sites and inhibits its ability to bind to TAR, to recruit CycT1 and CDK9 and to relocate to the nucleus.
   - The expression of the integrated HIV genome induces the production and activation of PKR activators such as IFN and p53. High levels of activated p53 and IFN stimulate the expression of PKR.
   - Direct interaction between Tat and p53 and inhibition of p53 proapoptotic signalling

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